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The carcinoma-associated mucin, MUC1, is a large transmembrane glycoprotein that over-expressed on >92% of primary and metastatic breast cancers. We created a mouse that lacks a functional *Muc-1* gene and demonstrated that mice developed normally. Mtag transgenic mice were bred onto the Muc-1 deficient and wild type backgrounds and tumor growth rates were compared. Mtag induced tumors grew significantly slower in Muc-1 deficient mice than in control mice. There were no differences in the rates of cellular proliferation or apoptosis in Mtag induced mammary tumors growing in Muc-1 deficient mice verses control mice. Depletion of NK cells resulted in acceleration of tumor growth rate in Muc-1 deficient animals but overall there were no consistent differences in tumor growth rate between Muc-1 deficient and wild type mice, irrespective of NK status. We demonstrated that mammary tumors induced by over-expression of the neu proto-oncogene in transgenic mice developed significantly faster in pure strain FVB mice than in F1 mice, suggesting that C57Bl/6 mice may contain tumor suppresser genes that inhibit tumor development

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Introduction:

Our goal is to understand the function of the tumor-associated mucin, MUC1, in the progression of cancer in the mammary gland. MUC1 is highly expressed by the majority of cancers and, in particular, by >92% of primary and metastatic breast cancers. The MUC1 protein is a large, rod-like molecule that projects far from the cell surface as a long filament. The protein core is extensively glycosylated through O-glycosidic linkage to serine and threonine, with as much as 50 to 90% of its molecular mass made up of oligosaccharide side chains. This contributes significantly to the rigidity of the molecule. MUC1 is expressed on normal epithelial tissues at low levels. Appearance of MUC1 correlates closely with epithelial differentiation in various organs and is detected well before the organs are functional. The presence of the large, highly extended molecule of MUC1 on the surface of epithelia suggests that it may act as a physical barrier protecting the cells. MUC1 may be involved in epithelial morphogenesis, perhaps acting to mask adhesive molecules present on the cell surface and aiding in the formation of a lumen. When epithelial tissues become cancerous, MUC1 expression is increased at least ten fold, and the glycosylation and spatial distribution of the protein at the cell surface is altered. In many cancer cells polarization of the epithelial cells is lost and the MUC1 protein can be detected on all cell surfaces, including those facing the stroma and adjacent cells. Under these circumstances, the anti-adhesive property of MUC1 may destabilize cell-cell and cell-substratum interactions, thus promoting the disaggregation of a tumor site, leading to tumor spread and metastasis. Previous *in vitro* studies have suggested various possible roles for the MUC1 mucin in facilitating tumor growth, including inhibition of cell-cell contacts, protection from recognition and destruction by immune cells, and also serving as an E-selectin ligand to facilitate escape of metastatic cells from the blood stream. Thus overexpression of the Muc1 molecule could provide many potential benefits to tumor cells. However, direct evidence of a role for MUC1 in the development and progression of breast cancer has not been demonstrated previously.

The ability to create mice that possess deficiencies in specific genes is providing important insights into the physiological role played by specific proteins during embryonic and postnatal development and during adult life. The expression pattern of the *Muc-1* gene in the adult and embryo of the mouse is similar to that of the human (the human gene designation is *MUC1*; the mouse gene is *Muc1*) (Braga et al., 1992). Muc1 expression is also elevated in mouse mammary gland tumors. Since mammary gland cancer in the mouse closely resembles human breast cancer and expression patterns are similar, our experiments should enable us to analyze the functional role of Muc1 in the development and progression of cancer. To investigate the biological function of the Muc1 protein we disrupted the *Muc1* gene using homologous recombination in mouse embryonic stem cells. Mice were generated that lacked expression of the Muc1 protein. We and others had postulated that Muc1 on the apical surface of differentiating epithelial cells may repel adjacent cells or mask adhesive molecules, thus promoting the formation of a lumen. However, we were surprised to find that, despite the widespread expression of Muc1 during epithelial organogenesis, mice lacking Muc-1 protein were born at the expected frequency and appeared normal in all respects. We have utilized the Muc1 deficient and wild type mice to investigate the role of the Muc1 molecule in normal development and in the development and progression of breast cancer in mice.

Methods and Materials

PCR Screening: PCR amplification was used to routinely identify mice with the desired genetic characteristics in the colony. PCR was carried out in a total volume of 50 µl in 200 µl PCR tubes with the following reagents: 5 µl lOX PCR buffer (Boehringer Mannheim, Indianapolis, IN), 0.02% formamide, 1 mM $MgCl_2$, 0.2 mM dNTPs, 100 nM of primers, l.25 units of Taq polymerase (Boehringer Mannheim), 1 µl of tail DNA (approximately 500 ng), and reagent quality H_20. The amplification program consisted of one cycle of ten min at 94°C and 40 cycles of 30s each at 94°C, 61°C and 72°C. The PCR product of each reaction was analyzed by size-fractionation through a 1% agarose gel.

	Primers	Annealing Temp	Product Size
Neu Primers	5'-CAGGTGCAAGCACTATTGACC-3'	58°C	550 bp
	5'-CTCAGAGCTCAGATCAGAACC-3'		
Muc1 Primers	5'-TCCCCCCTGGCACATACTGGG-3'	62°C	262 bp
	5'-ACCTCACACACGGAGCGCCAG-3'		
LacZ Primers	5'-TCCCCCCTGGCACATACTGGG-3'	62°C	262 bp
	5'-TTCTGGTGCCGGAAACCAGGC-3'		
Mtag Primers	5'-AGTCACTGCTACTGCACCCAG-3'	61°C	555 bp
	5'-CTCTCCTCAGTTCTTCGCTCC-3'		

Immunohistochemistry: Tissues were fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid), paraffin embedded, and sectioned for immunohistochemical analysis.

Muc1 Staining: Briefly, endogenous peroxidase activity was decreased by incubating the tissue sections with 0.6% H_2O_2 in PBS (pH 7.4) for 30 minutes at 25°C. After washing with PBS pH 7.4, sections were blocked with 50% fetal calf serum and stained with CT1 rabbit polyclonal antibody to the cytoplasmic tail of Muc1 or antibody previously blocked with 5mg/ml of synthetic peptide (diluted 1:50) for 1 hr at 25°C. Following three 5 minute washes with PBS pH 7.4, the sections were incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit antibody (Dako Corporation, Carpinteria, CA, diluted 1:100) for 1 hr at 25° C. Sections were washed three times for 5 minutes each and coverslipped.

Neu Staining: Briefly, endogenous peroxidase activity was decreased by incubating the tissue sections with 0.6% H_2O_2 in PBS (pH 7.4) for 30 minutes at 25°C. After washing with PBS pH 7.4, sections were incubated in 1X antigen retrieval buffer at 95°C for 5 minutes. Sections were then incubated in 0.05% saponin (Sigma) in distilled water for 30 min at 25°C . After washing with PBS pH 7.4 3 times, sections were blocked with 50% fetal calf serum and stained with Ab-3 murine monoclonal antibody that recognizes the *neu* oncogene (Oncogene Science,Uniondale, NY; diluted 1:100), for 1 hr at 25°C. Following three 5 minute washes

with PBS pH 7.4, the sections were incubated with peroxidase-conjugated rabbit anti-mouse antibody (Dako Corporation, Carpinteria, CA, diluted 1:50) for 1 hr at 25°C. Following three 5 min washes, the substrate solution consisting of 0.03% hydrogen peroxide in PBS and 1 mg/ml diaminobenzoate (Sigma) was added, and the color allowed to develop for 5-8 min. Following color development, the reaction was quenched by washing the slide in distilled water. The tissue sections were counterstained lightly with hematoxylin.

Mammary Gland Whole Mount Analysis: Muc-1 deficient and wild type mice inbred on a C57Bl/6 background were terminated at 4, 5 and 6 weeks of age. All animals were housed in close proximity to allow synchrony of estrous cycles. Auxiliary and inguinal mammary glands were removed, spread on glass slides and fixed in acetone overnight. The tissues were cleared by passage through xylene and a graded series of alcohols (100, 95, 80, 70 and 40%). The tissues were subsequently stained for 20 min in Mayer's hematoxylin and destained in ammonium water (3.4mM NH_4OH). To analyse the extent of mammary gland development, tissues were photographed under a dissecting microscope, the images scanned into the computer and the percentage of the total mammary fat pad occupied by ductal elements was measured.

Northern Analysis: Total RNA was isolated from tissues dissected from +/+, +/- and -/- littermates, homogenized in guanadinium isothiocyanate/β-mercoptoethanol solution followed by centrifugation through a CsCl gradient. RNA (15 ug) was separated through a 1.2% formaldehyde gel, transferred to Hybond-N nylon membrane (Amersham) and stained with methylene blue solution to detect 18 and 28 S robosomal RNAs prior to hybredization. Blots were hybredized overnight with mouse Muc1 cDNA probe pMuc2TR labeled by random priming in the presence of [α-^{32}P]dCTP (Amersham) to a specific activity of >1 x 10^8 dpm/μg.. Subsequent washing procedures were carried out according to the manufacturer's specifications.

Gene Expression Analysis: RNA samples isolated from tissues of +/+, +/- and -/- littermates were investigated for the expression of a variety of genes utilizing a slot-blot approach. 1ug of total RNA from +/+, +/- and -/- tissues was loaded into adjacent wellls of a vacuum slot-blot apparatus (Schleicher and Schuell) and vacuum-blotted onto nylon membrane (Hybond-N) under

conditions recommended by the manufacturer. Duplicate blots were prepared and hybredized with probes to a large panel of genes (Table 1). Genes that were identified as being potentially up-regulated were investigated by Northern analysis. Antisense oligonucleotide probes were 5' end-labeled with [γ^{32}P]ATP (Amersham) utilizing T4 polynuleotide kinase (New England Biolabs, Beverly, MA). All probes were hybredized as described previously for Northern analysis.

Role of Muc1 in Mammary Tumor Development: To study the role of Muc1 in mammary tumor development, mice transgenic for the polyoma virus middle T antigen oncogene under control of theMMTV 3'LTR promoter element (Mtag) were crossed with outbred mice (129sv x C57Bl/6) mice homozygous for the mutant or wild type Muc1 allele. Mice were weaned at 3 weeks of age and housed in groups of 4-5 mice per cage. Animals were genotyped by PCR using a small snip of tail as a DNA source. Male mice that were positive for the Mtag transgene were selected and bred with outbred mice (129sv x C57Bl/6) mice homozygous for the mutant or wild type Muc1 allele. Mice were again screened by PCR to identify female animals that were positive for the Mtag transgene and homozygous for either the mutant or the wild type Muc1 gene. These animals were housed in groups of 5 and palpated on a weekly basis for the duration of the study.

Tumor Measurement: For all tumor growth studies, female mice were weaned at 3 weeks of age and housed in groups of 4 to5 animals per cage. Mice were palpated on a weekly basis for the appearance of tumors. Once tumors appeared tumor growth was measured with calipers and tumor weight was calculated as follows:

$$g = \frac{(l \times w^2)}{2}.$$

Cell Proliferation and Apoptosis: To study cell proliferation rates and the rate of apoptosis, 20 female mice transgenic for the polyoma virus middle T antigen were produced on the Muc-1 deficient or wild type backgrounds (n= 10 per group). For the experiment, mice were palpated twice weekly from day 60 and were terminated when the tumors reached a weight of 1 gram. On termination day, mice were injected i.p. with 1.5 mg/mouse of 5' Bromodeoxyuridine (BrdU,

Sigma, St. Louis, MO). Mice were terminated 2 h post injection and tumors were removed, fixed in methacarn for 2 h and stored in 70% ethanol at 4°C. Tumors were parafin embedded and 5uM serial sections were cut.

To measure cell proliferation, tumors were stained for BrdU incorporation. Slides were deparaffined, treated with a mild enzymatic digest (0.005% pepsin in 0.025M HCl for 15 min at RT) then blocked in 50% fetal calf serum (Gibco, Grand Island, NY). The DNA was denatured by treatment with 2N HCl (60 min at 37°C). Sections were treated with anti-bromodeoxyuridine primary antibody (Boehringer Mannheim, Indianapolis, IN) followed by a peroxidase-conjugated anti-mouse secondary antibody (Dako, Carpinteria, CA). The tissues were then exposed to diaminobenzoic acid for approximately 1 to 2 minutes and counterstainded with hematoxylin. Cells staining positive for BrdU and total cells were enumerated for 5 randomly chosen fields at 400X magnification using a 25 square 1mm^2 reticule. Control sections treated with an irrelevant primary antibody failed to exhibit significant nuclear staining for any of the tissues tested. Liver sections exhibited proliferation rates of less than 1%.

To measure cellular levels of apoptosis and necrosis, tumor sections were stained using the TUNEL assay. Slides were deparaffined, treated with a mild enzymatic digestion (0.004% pepsin in 0.025M HCl for 15 min at RT) and stained with the in situ cell death detection kit (Boehringer Mannheim). The level of apoptosis in each specimen was rated on a 4 point scale and all observations were confirmed by two independent observers. Liver sections did not exhibit significant levels of apoptosis. Similarly, tumor sections labeled in the absence of the terminal deoxynucleotide transferase enzyme failed to stain for apoptosis.

In Vitro Tumor Proliferation: To investigate the ability of tumor cells to proliferate under conditions in which oxygen and nutrient concentrations were not growth limiting, cells from middle T antigen induced mammary tumors growing in Muc-1 deficient and wild type mice were placed into long term tissue culture. Muc1 deficient and wild type mice (3 mice per group) were terminated, their tumors removed aceptically, finely minced and dissociated in trypsin for 5 min at 37°C on a shaking platform. Cells were plated in DMEM containing 10% fetal calf serum and

passaged for at least 10 generations. For the growth rate experiments, cells were plated in 6 well plates at a concentration of 5 X 10^4 cells per well in DMEM containing 10% fetal calf serum. The cells were incubated at 37°C, 10% CO_2 and the media was changed every 4 days. To evaluate cell proliferation, cells were detached from the dish by a 5 min treatment with versine at 37°C followed by a 2 min exposure to trypsin at 37°C. Cells were counted on a hemocytometer on a phase contrast microscope. Cell counts were performed in triplicate and the experiment was repeated 3 times with similar results.

In Vivo Depletion of Natural Killer Cells: For the study, outbred (C57Bl/6 x 129sv) Muc-1 deficient and wild type male mice containing the MTag transgene were crossed to Muc-1 deficient or wild type C57Bl/6 females to ensure that all experimental mice were at least 50% C57Bl/6. It was important that all mice be at least 50% C57Bl/6 as the epitope recognized by the NK1.1 antibody is only expressed on C57Bl/6 mice. Mice were weaned at 3 weeks of age and randomly assigned to either NK1.1 or vehicle-injected groups (n = 20 per group) and housed 4 - 5 mice per cage. Mice were injected ip with 150 ul of ascites (diluted 1:1 with sterile PBS) or PBS at weekly intervals beginning at 4 weeks of age.

Natural Killer Cell Assay: Mice were injected ip with either NK1.1 antibody or PBS 3, 5 or 7 days prior to the assay. For the study of long term suppression of NK cell activity, mice were injected in NK1.1 antibody on a weekly basis from 4 until 17 weeks of age. Mice were terminated 3 days following the final injection.

Eighteen hours before the assay mice were injected i.p. with 100ug of polyinosinic:polycytidylic acid (Sigma, St. Louis, MO) to stimulate NK cell activity. For the assay, mice were terminated, spleens aseptically removed and dissociated in RPMI medium (Grand Island Biochemical Co., Grand Island, NY). Red blood cells were lysed using Tris-NH_4Cl. Viable cells were counted and resuspended in RPMI supplemented with 10% fetal bovine serum (Gibco) and 10 mM HEPES (Sigma) at a concentration of 1.5 x 10^7 cells/ml of medium. Yac-1 lymphoma cells were labeled with 500 uCi sodium [^{51}Cr] chromate/10^7 cells (Amersham, Arlington Heights, IL) for 90 min, washed and resuspended at 10^5 cells/ml of medium. Cell suspensions of 100 ul were added to 96 well V-bottomed plates (Rainin Instrument

Co., Woburn, MA). Spleen cells were added to each well to produce effector to target cell rations of 150:1, 75:1, 37.5:1, 17:1, 9:1, 4.5:1. Plates were incubated for 6h at 37°C in 95% air, 5% CO_2 and [^{51}Cr] release was from lysed target cells was determined by gamma counting. The percent of specific [^{51}Cr] released at each effector to target cell ratio was computed using the formula:

$$\% \text{ Specific Lysis} = \frac{\text{Test cpm - Spontaneous cpm}}{\text{Total cpm - Spontaneous cpm}} \text{ X } 100.$$

Where Test cpm = counts in experimental cultures of target cells and effector cells.

Spontaneous cpm = counts in cultures containing only target cells.

Total cpm = counts obtained by adding 100 ul of 1N HCl to target cells to lyse all cells.

Effect of Genetic Background on neu Protooncogene-Induced Tumorigenesis: To insure that genetic background did not affect neu-induced mammary tumor growth, tumor growth was compared between FVB (n=11) and F1 (FVB x C57Bl/6; n=43) neu transgenic mice. *Neu* transgene positive female mice were housed in groups of 4-5 mice per cage and palpated biweekly beginning at 7 months of age. To further investigate the effect of genetics on neu-induced mammary tumor latency, F1 mice were backcrossed onto either FVB or C57Bl/6 mice and tumor growth was followed in resulting *neu* positive female offspring (n = 102 and 50, respectively).

Western Blotting: Mammary tumor tissues were rapidly frozen in liquid nitrogen and stored at -80°C until use. Tissues were lysed and sonicated (Branson Microtip,) in a solution comprising 0.05M sodium chloride, 0.02M Tris HCl pH 7.4, 100ug/ml leupeptin, 50ug/ml aprotinin and 1% NP-40 (all reagents were from Sigma). Equivalent amounts of protein were separated onto 5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond C++,). Membranes were stained with ponseau S and photographed. Nitrocellulose membranes were blocked in 5% non-fat dry milk in PBS + 0.05% Tween 20 and probed with either anti-neu antibody (Ab-3, Oncogene Science, diluted 1 in 2500) for 1 hr at 25°C. Membranes were

incubated with 2° antibody (HRP conjugated rabbit anti-mouse antibody, Dako, diluted 1 in 2000) for 1 hr at 25°C. The membranes were visualized by ECL (Amersham). Membranes were stripped for 1 hr in 1X PBS and blotted with anti-actin antibody (CP-01, Oncogene Research, Cambridge, MA, diluted 1 in 1000) as described.

Statistics: Latencies of *neu*-induced mammary tumor development were plotted using Kaplan-Meier survival plots and compared by Chi-squared analysis. The number of unlinked genetic controlling *neu*-induced tumor latencies of mammary tumors induced by the over-expression of the *neu* proto-oncogene was estimated using the method of Dietrich et. al. (Dietrich et al., 1993; Wright, 1968). This method assumes that the average of the mean latency for the F1 and the FVB mice equals the mean latency for the F1B1 mice and that the distributions be normally distributed with equal variances. Our data for tumor latency meet these criteria.

Results

Specific Aim 1: Analysis of the effects of Muc-1 gene mutations on epithelial organogenesis.

Mice homozygous for the Muc-1 mutation have been produced and have been demonstrated to be healthy, fertile and viable (Spicer et al., 1995). Over 400 offspring of heterozygous intercrosses were screened in outbred mice (C57Bl/6 x 129SV). Homozygous animals were identified through a PCR-based screening procedure and results were initially confirmed through Southern blotting of EcoRI-digested tail DNA utilizing the 5' flanking probe. In all cases, animals homozygous for the disrupted Muc-1 allele (-/-) were obtained at the expected Mendelian frequency of 1:2:1 (Wild type : Heterozygotes : Homozygous Mutants). In addition, inbred 129SV heterozygotes were obtained from the original chimeric animals and intercrossed to derive an inbred line homozygous for the Muc-1 mutation. Similarly, inbred C57Bl/6J heterozygotes are being derived through a series of backcrosses onto C57Bl/6J. These mice are currently at N8 (99% inbred with respect to C57Bl/6J). Homozygous Muc-1 mutant mice of the129SV and C57Bl/6J line (N8) have been produced and appear healthy and viable.

In order to determine whether the insertion of the LacZ-pgk neo cassette resulted in the efficient disruption of Muc-1 transcription and subsequent translation, total RNA was prepared from a panel of tissues isolated from +/+, +/- and -/- litter mates. Approximately equivalent amounts of total RNA were subjected to northern analysis with the previously characterized Muc-1 cDNA probe, pMuc2TR (Spicer et al., 1991). Expression of Muc-1 was found to be reduced in the tissues of heterozygous mice and undetectable in homozygous mice (Fig 1). In addition, immunohistochemistry indicated no detectable Muc-1 protein on the apical surface of Muc-1 -/- secretory epithelial tissues (Fig 2). Thus, targeted inactivation of the Muc-1 gene by the replacement vector, 129Muc-1GT, resulted in the creation of a null Muc-1 allele.

Mice deficient in Muc-1 were obtained at the expected frequency from all crosses. These mice appeared to develop normally and gained weight at the same rate as their heterozygous and wild-type litter mates (data not shown). Examination of hematoxylin-eosin stained sections prepared from all the major organs revealed no obvious differences between Muc-1 deficient mice and their corresponding litter mates (data not shown). Similarly, whole mounts of virgin mammary glands of twelve week old wild-type and Muc-1 null animals showed no obvious differences in glandular morphology (data not shown). All possible pairwise crosses of genotypes indicated no differences in the fertility of the parents, subsequent litter size, growth rate and survival of the litters (data not shown). This would suggest that Muc-1 present in milk is not important for the growth and survival of neonates under pathogen-free conditions.

We explored the possibility that the up-regulation of expression of one or more mucin-like genes or membrane glycoproteins may have accounted for the apparent lack of a phenotype in Muc-1 deficient mice. Probes were obtained either as antisense oligonucleotides (50mers) or, alternatively, cloned cDNAs were utilized (Table 1). Total RNAs isolated from +/+, +/- and -/- litter mates were investigated by slot blot analyses with the various probes. No difference in expression levels was observed for Muc-2, or Muc-4, although high levels of Muc-4 expression were observed in lactating mammary gland, salivary gland, lung, stomach, kidney, and colon. Similarly, no difference was observable for other mucin-like genes, including ASGP-2, CD34, CD43 (leukosialin), glycophorin and MadCAM-1. The expression level of GlyCAM-1 was

elevated in several outbred homozygous animals, but this apparent increase in expression was not consistent in inbred homozygotes, nor did it appear to correlate with an increase in GlyCAM-1 protein levels in milk. In addition, no difference was observable in the expression of thrombospondin-3 (Thbs-3), although the expression of this gene did appear to be highly variable in the tissues tested. This is important as Thbs-3 is located only 2.5 Kbases upstream from the Muc-1 gene and its' expression could potentially have been altered by the mutation of Muc-1.

Although lack of the Muc-1 molecule did not appear to significantly affect the development, viability or fertility of homozygous Muc-1 mutant mice, it is possible that there are subtle defects in organogenesis. To address this possibility, mammary gland organogenesis in Muc-1 deficient and control animals was studied. The mammary gland is an organ system which develops late, maturing in the juvenile mouse and it is not required for survival of the individual. Thus it is an excellent system in which to investigate possible subtle changes in organogenesis in Muc-1 deficient mice. Preliminary experiments suggested that ductal elements developed more rapidly in Muc-1 deficient mice than in wild type control mice, but that similar levels of ductal development were achieved in adult mice of both groups. However, the small sample sizes (n=3 per group per time point) in this trial may not be representative of the total population. Thus a larger study was undertaken to investigate the role Muc-1 in the development of the mammary gland. For the study, virgin female C57Bl/6 mice of the Muc-1 deficient and wild type strains were terminated at 4, 5 and 6 wk of age. Inguinal and auxiliary mammary glands were removed, prepared for whole mount staining and the extent of ductal invasion of the mammary pad was evaluated. At four weeks of age, ductal elements occupied a greater percentage of the mammary fat pad in Muc-1 deficient mice compared with wild type mice (Figure 2, $p<0.01$). By five and six weeks of age there were no significant differences in the extent of invasion of the mammary fat pads by epithelial elements (Figure 3).

Specific Aim 2: Analysis of the effects of Muc-1 gene mutation on tumor formation and progression.

To investigate the role of Muc-1 in tumor development and/or progression, we compared the growth of oncogene-induced mammary tumors in Muc-1 -/- and +/+ mice transgenic for the polyoma virus middle T antigen. The middle T antigen under the control of the mouse mammary tumor virus (MMTV) promoter demonstrates specific expression in the mammary gland and to a lesser extent the salivary gland (Guy et al., 1992a). Virgin female mice of this strain have been shown to develop multifocal breast tumors by 2 months of age and by 4 months of age greater than 50 percent of these mice develop lung metastases. Interestingly, the middle T antigen has been demonstrated to require the presence of the src oncogene for its ability to transform mammary cells (Guy et al., 1994). Similarly, it has been demonstrated that the neu oncogene, implicated in up to 30 percent of human breast cancers (Slamon et al., 1987), binds to and activates src tyrosine kinase activity (Muthuswamy et al., 1994). We employed the middle T oncogene due to its rapid time course of tumor induction, reliable production of spontaneous tumor metastases, and the possible commonalty of signal transduction pathways with the neu protooncogene.

For the study, 85 female Muc-1 -/- mice and 35 female Muc-1 +/+ mice were utilized. All mice were virgin females positive for the polyoma virus middle T antigen transgene. Mice were housed in groups of 5 mice per cage and palpated 3 times per week, starting at 60 days and continuing through 124 days. Fifty percent of mice in this study developed palpable lesions of the mammary gland by 68 days of age. There was no significant difference in the rate of appearance of palpable lesions between Muc-1 mutant and wild type mice. By 4 months of age, tumors appeared in 100% of wild-type mice and in 98% of mutant mice. Tumors in Muc-1 mutant and wild type mice had similar histological appearances and were poorly differentiated adenocarcinomas (Fig. 4A). Pathological analysis showed that the tumors were high grade, based on the high mitotic rate, the solid growth pattern, and the presence of central necrosis. Immunohistochemical analysis using antiserum directed to the Muc-1 cytoplasmic tail showed that tumors that developed in Muc-1 +/+ animals expressed high levels of Muc-1 (Fig. 4B).

Interestingly, tumor growth rate differed significantly between the two groups (Fig. 4C). As early as 104 days of age, Muc-1 -/- mice had significantly smaller tumors than did mice with wild type Muc-1 alleles ($p< 0.05$) and by the 124 day endpoint, differences in tumor size were highly significant ($p<0.001$) (two-sample t-test). These studies are the first to directly implicate the Muc-1 molecule in the facilitation of mouse mammary tumor growth.

Overexpression of Muc-1 facilitates tumor growth of polyoma virus middle T antigen induced mammary tumors. However, the mechanism by which Muc-1 overexpression facilitates tumor growth is not clear. It is possible that Muc-1 overexpression may increase the rates of cellular proliferation or decrease rates of cell death. An effect of Muc-1 on rates of cell proliferation and/or death could be the result of Muc-1 overexpression blocking cell-cell contact and thus preventing the transmission of contact mediated growth inhibitory signals from surrounding normal cells. It has been demonstrated that MUC1 overexpression in cell lines can block cell-cell and cell-substratum contacts (Ligtenberg et. al., 1992; Wesseling et. al., 1995). Alternately, Muc-1 may be involved in signal transduction in tumor cells. The cytoplasmic tail of Muc-1 is 88% conserved in all mammalian species tested and it contains 7 tyrosine residues, 6 of which are conserved (Spicer et. al., 1995). Recently it has been demonstrated that MUC1 is phosphorylated *in vivo* on tyrosine and serine residues and that phosphorylated MUC1 may bind signal transduction molecules through SH2 domains (Zrihan-Licht et. al., 1994; Pandey et. al., 1995).

To investigate the role of Muc-1 overexpression in tumor cell growth, we measured rates of cellular proliferation, apoptosis and levels of necrosis in tumors from Muc-1 deficient and wild type control mice. Further, to ensure that nutrient or oxygen levels within the tumor were not limiting growth rate of tumor cells, rates of proliferation of tumor cells derived from polyoma virus middle T antigen induced mammary tumors growing in Muc-1 deficient and wild type mice were measured *in vitro*. There were no differences in rates of cellular proliferation (Figure 5), apoptosis or levels of necrosis (Figure 6) between mammary tumors from Muc-1 deficient and wild type control mice. Further, *in vitro* rates of cellular proliferation did not differ significantly between mammary tumors from Muc-1 deficient and wild type control mice (Figure 7).

One possible mechanism by which the overexpression of Muc-1 facilitates mammary tumor growth could be by blocking the ability of immune effector cells to recognize and lyse tumor cells. This hypothesis is supported by the findings of Wiel-van Kemenade et al. (1993), who demonstrated that tumor cells transfected to overexpress MUC1 were resistant to lysis by both Natural Killer (NK) cells and cytotoxic T lymphocytes compared to non-transfected parental cells. To investigate if increased NK cell recognition and lysis of tumor cells in Muc-1 deficient mice was responsible for the slower tumor growth rates observed in Muc-1 null mice in our model, NK cell activity was depleted by repeated injections of the NK1.1 antibody (Seaman et al., 1987). A single injection of NK1.1 antibody resulted in complete loss of NK cell activity for at least 7 days (Figure 8A). Repeated injections of NK1.1 antibody at one week intervals resulted in the sustained abrogation of NK cell activity, as indicated by the lack of NK cell activity in mice after 3 months of injections (Figure 8B). For the study, Muc1 null or wild type mice containing the Mtag transgene were weaned at 3 weeks of age to groups of 4 to 5 mice. Beginning at 4 weeks of age, mice were injected with NK1.1 antibody or PBS (n=20 per group). Animals were palpated on a weekly basis beginning at 6 weeks of age. Tumor growth in animals treated with NK1.1 antibody did not differ significantly compared with animals injected with vehicle alone in either Muc-1 deficient or wild type mice (Figure 9, $p>0.2$). Further, there was also no difference in tumor growth rates between Muc-1 deficient and wild type mice in the vehicle-injected conditions. Thus the vehicle-injected groups failed to replicate the finding that tumors grow significantly more slowly in Muc-1 deficient mice than in wild type mice. Further, variability within a group was significantly greater than variability between groups. These findings suggested that MTag-induced tumor growth rate could be affected by the genetic background of the animal. Genetic influences appeared to have a greater affect on tumor growth rate than did treatment with NK1.1 antibodies, making it difficult to analyze the role of NK cells in mediating the decrease in tumor growth rate observed in Muc-1 deficient mice.

To investigate the effect of genetic background on MTag-induced tumor growth rate, pure strain FVB males containing the MTag transgene were crossed onto FVB, C57Bl/6 or 129sv females. Tumor growth rates were followed for 80 days in the resulting MTag transgene positive

female offspring. MTag-induced tumor growth rates were not significantly different in pure strain FVB mice(n=22) and FVB x 129sv F1 mice (n=31) (Figure 10), while tumors in FVB x C57Bl/6 F1 mice (n=11) grew significantly slower than those in either FVB or FVB x 129sv F1 mice (Figure 10, $P<0.01$). Thus C57Bl/6 mice contain genetic elements that significantly suppress tumor growth induced by the expression of the MTag transgene. The effect of C57Bl/6 genome on MTag-induced tumor growth could have serious implications for studies of the role of Muc-1 overexpression in MTag-induced mammary tumors. The initial study of Muc-1s role in MTag-induced mammary tumors used outbred (C57Bl/6 x 129sv) mice and, although every attempt was made to ensure that Muc-1 deficient and wild type control mice had equivalent genetic backgrounds, the results of the study could have been influenced by variations in genetic background of the mice. To examine this issue, all animals in the study that exhibited rapid tumor growth rate patterns indicative of the pure strain FVB (or 129sv) genetic background were removed and the data was reanalyzed. Animals were removed from the data subset if their tumor displayed signs of rapid early growth; tumor burden exceeded 0.3 grams at 90 days of age (11 Muc-1 deficient mice and 8 wild type mice). On reanalysis of the data, tumor growth was still significantly slower in Muc-1 deficient mice than in wild type control mice at 125 days of age (Figure 11, $p<0.01$). Thus we conclude that the initial observation that the overexpression of Muc-1 significantly facilitates tumor growth is valid.

Specific Aim 3: Analysis of the effect of Muc-1 gene deletion on metastasis of mammary gland tumors.

To investigate whether the Muc-1 molecule plays a significant role in facilitating tumor metastasis, Muc-1 mutant and wild type mice were crossed with transgenic mice expressing the polyoma virus middle T antigen under the control of the mouse mammary tumor virus promoter. As mentioned previously, 85 Muc-1 -/- female mice and 35 Muc-1 +/+ female mice were examined. Mice were housed in groups of 5 and palpated 3 times per week from day 60 to day 124. Mice were terminated on day 124 and their organs examined for the presence of metastases. As was previously reported (Guy et al., 1992a), only the lungs contained metastatic tumor foci. At termination, the lungs were removed, fixed in methacarn and scored for grossly observable

lung metastases under the dissecting microscope. Overall, 58% of mice developed grossly observable lung metastases, with 53% of Muc-1 -/- mice and 67% of Muc-1 +/+ mice developing metastases (Fig. 12). Although this difference suggests a trend towards decreased rates of tumor metastasis in Muc-1 -/- mice, it was not statistically significant as assessed by chi-square analysis ($p > 0.10$). However, based on the sample sizes in this study, the power to statistically detect the observed difference was only 33%. It is possible that with a larger sample size, this difference in metastatic rate would be statistically significant.

In previous a study, the overexpression of Muc-1 on MTag-induced mammary tumors resulted in a trend towards increased lung metastasis compared with metastasis in Muc-1 deficient mice (67% vs 53%, $p>0.10$). With 35 animals in the control group and a 14% difference in metastasis between the two groups, the power to statistically detect such a difference is only 33%. Thus, the data suggest that Muc-1 overexpression could facilitate tumor metastasis, but further research is required to substantiate this difference. As was mentioned previously, one potential role of Muc-1 overexpression in tumors is to shield the tumors from recognition by the immune system. It has been demonstrated that NK cells play an important role in controlling metastatic spread and it has been shown that overexpression of Muc-1 in vitro decreases the ability of NK cells to recognize and lyse tumor cells. Thus it is possible that one role of Muc-1 overexpression is to protect tumors cells from lysis by NK cells during the metastatic cascade. To test this hypothesis, rates of metastasis were compared between Muc-1 deficient and wild type control mice treated with either anti-NK antibodies (NK1.1) or vehicle (n=20 mice per group). Depletion of NK cell activity did not result in a change in the rate of metastasis in either Muc-1 deficient or wild type mice (Figure 13). This finding could suggest that NK cells do not play a significant role in limiting the metastasis of MTag induced mammary tumors. However, as discussed above, the animals in this study are of an outbred genetic background and it is possible that the outbred background affected the rate of lung metastasis independent of NK cell activity.

Specific Aim 4: Analysis of the Effect of Genetic Background on *neu* Protooncogene-Induced Mammary Tumorigenesis.

To insure that the effect of Muc-1 overexpression was not specific to tumors induced by the polyoma virus middle T antigen oncogene, we wished to investigate a second oncogene-induced tumor model to study the effect of Muc-1 gene mutation on tumor development and progression. The neu transgenic mouse is a useful model for several reasons. It is estimated that 30 percent of human breast cancers overexpress the neu protooncogene (Slaman et. al., 1987). Further, neu proto-oncogene transgenic mice have long tumor latencies and develop focal mammary adenocarcinomas surrounded by hyperplastic mammary epithelium similar to that observed in humans (Guy et. al., 1992a&b). Thus the *neu* transgenic mouse would be an ideal model in which to investigate the role of Muc1 in mammary tumor development.

As the neu transgenic mice were on an FVB background and the Muc1 mutant mice were on a C57Bl/6 background, a pilot study was undertaken to insure that the genetic background of the mice did not significantly affect tumor growth in this model. We demonstrated that genetic background significantly affects mammary tumor induction in transgenic mice that overexpress the unactivated *neu* protooncogene in the mammary gland under the control of the MMTV 3'LTR promoter. Tumor latency was significantly increased in FVB x C57Bl/6 F1 (F1) mice compared with pure strain FVB mice (Figure 14A, $p<0.001$). To further investigate this phenomina, F1 mice were backcrossed onto FVB and C57Bl/6 mice. Tumor latency in [FVB x C57Bl/6] F1 x FVB backcross mice (F1B1) was intermediate between that observed in pure strain FVB and F1 mice and was significantly different from both groups (Figure 14A, $p<0.001$). Tumors were not observed in any [FVB x C57Bl/6] F1 x C57Bl/6 mice by 2 years of age (n=50). Analysis of the distribution of tumor latencies in the 3 groups of animals (Figure 14B) allowed an estimate of the number of genetic loci involved in modulating *neu*-induced mammary tumorigenesis. Development of mammary tumors in FVB and F1 mice was only affected by environmental variance as the animals within a group are genetically identical, while tumor latencies in the F1B1 mice were affected by both genetic and environmental variance as allelic differences are segregating in these mice. The data suggest that genetic variance accounts for

approximately 88% of the total variance in tumor latency in the F1B1 mice. Analysis of the genetic variability in latency for *neu*-induced mammary tumors suggests that 2.7 independent loci may be involved in modulating *neu* induced tumorigenesis.

To investigate the possibility that the altered tumor latencies observed in this study could result from differences in the level of neu expression, neu expression levels were compared in the mammary glands of virgin female FVB and F1 *neu* transgenic mice at three months of age. Neu protein was expressed in the epithelial elements of mammary glands in both FVB and F1 mice (Figure 15). The expression of the neu proto-oncogene was variable, with some cells expressing strongly, while others expressed the protein weakly or not at all. Interestingly, variations in the levels of neu expression were greater in cells from a single mouse than they were between mice of the FVB and F1 strains.

Although tumor development was significantly delayed in F1 mice, it was of interest to investigate if the C57Bl/6 genotype also affected tumor growth rate. Interestingly, when tumor growth rates were compared, correcting for the effect of tumor latency, tumors developing in F1 mice grew faster than tumors developing in pure strain FVB mice. To directly compare tumor growth rate in these two groups, the data was logarithmically transformed and a best fit linear regression was estimated for each tumor. When the slopes of these lines were compared, tumors in F1 mice grew significantly faster than tumors growing in pure strain FVB mice (Figure 16, $p<0.01$).

To investigate the possibility that the increased tumor growth rates observed in F1 mice were due to altered levels of expression of the *neu* oncogene, tumors from both groups were stained for the presence of neu. Tumors derived from both FVB and F1 mice stained intensely for the neu oncogene. To further quantitate the level of neu expression in these tumors, western blots were performed using anti-neu antibodies. Once again there were no significant differences in the levels of neu expression between tumors derived from FVB and F1 mice (Figure 17).

Conclusions:

We have demonstrated that mice homozygous for the Muc-1 mutation are healthy, fertile and do not exhibit any deleterious effects in two different strains of mice. The present studies suggest that *Muc-1* mutation may alter the organogenesis of the mammary gland, as there was greater penetration of ductal elements into the mammary fat pads in Muc-1 deficient mice at four weeks of age. Currently the importance of the increased mammary gland development in Muc-1 deficient mice is unclear. The difference in mammary gland development between wild type and Muc-1 deficient mice is no longer apparent by 5 weeks of age, suggesting that it is of little functional consequence for the mice. It may reflect an earlier start or increased rate of organogenesis in the mammary glands of Muc-1 mutant mice.

These studies demonstrate for the first time that the Muc-1 molecule facilitates growth of breast tumors in mice transgenic for the MTag oncogene. Tumor growth rate was significantly decreased in mice homozygous for the Muc-1 mutation when compared with wild type control mice. Interestingly, the tumors did not exhibit different rates of proliferation or apoptosis, as measured by BrdU incorporation and the tunel assay respectively. This was confirmed by the finding that in vitro proliferation of Muc1-expressing and deficient tumor cells did not differ. These findings suggest that facilitation of tumor growth induced by the overexpression of the Muc-1 molecule by mammary tumor cells does not involve large changes in rates of cellular proliferation or apoptosis. However, it is possible that small changes in the rates of cellular proliferation and/or apoptosis which are below the sensitivity of the current assay system to detect could result from the overexpression of Muc-1 by mammary tumor cells. Such subtle changes in cell growth or death rates could be sufficient to account for the observed differences in tumor growth observed in this model given the exponential nature of tumor growth.

Another potential mechanism by which Muc-1 overexpression could affect tumor development and metastasis is by modulating the immunogenicity of mammary tumor cells. It has been demonstrated that the overexpression of MUC1 *in vitro* by transfecting tumor cells to express high levels of MUC1 protein results in protection from lysis by natural killer cells and

cytotoxic T lymphocytes (Wiel-van Kemenade et. al., 1993). It has been speculated that the presence of the large negatively charged MUC1 molecule on the tumor cell surface blocks access of immune cells to tumor specific antigens and other cell adhesion molecules present on the tumor cell surface. Thus it is possible that overexpression of Muc-1 by mammary tumor cells facilitates tumor growth by blocking tumor recognition and lysis by natural killer cells and cytotoxic T lymphocytes. To investigate the potential role of Muc-1 overexpression in protecting tumor cells from destruction by the immune system, we have studied the role of NK cells. We have demonstrated that injection of the monoclonal antibody NK1.1 on a weekly basis can maintain suppression of NK cell activity for a three month period. Maintained suppression of NK cells over the 3 months that tumors were growing in wild type and Muc1 deficient mice failed to significantly affect tumor growth rate in either group. However, studies into the role of NK cells in decreasing tumor growth rate in Muc-1 deficient mice were hampered by the effects of genetic background on MTag-induced mammary tumors and this makes interpretation of the results difficult.

We have demonstrated that induction of mammary tumors by overexpression of the *neu* proto-oncogene was significantly affected by genetic elements of C57Bl/6 mice in a dominantly acting fashion. This suggests that C57Bl/6 mice may contain tumor suppresser genes that affect mammary tumorigenesis induced by the *neu* proto-oncogene. We have recently reported that *neu* induced mammary tumors developing in FVB x C57Bl/6 mice undergo loss of heterozygousity at chromosomes 3 and 4 with high frequency (Ritland et al., 1997). Further, there is preference for the loss of the B6 allele in 78% of the LOH cases studied. Together data these suggest that the C57Bl/6 mice contain one or several tumor suppresser genes that regulate mammary tumor induction by over-expression of the *neu* proto-oncogene.

Alternately, it is possible that the MMTV 3'LTR promoter used to drive mammary specific over-expression of the *neu* proto-oncogene in these transgenic mice was less efficient at inducing gene expression in C57Bl/6 mice compared with FVB mice. If the MMTV 3' LTR were less active in C57Bl/6 mice, then F1 mice would express lower levels of the neu proto-oncogene in their mammary glands and could be at lower risk for developing mammary tumors. Recently it

has been reported that ras expression in MMTV-ras transgenic mice correlates with MMTV 3' LTR promotor methylation status and that the long tumor latencies observed in these mice are associated with an age dependent demethylation of this promoter (Mangues et al., 1995). Further, studies have demonstrated that mice have strain specific modifiers of methylation and that C57Bl/6 mice are more efficient at methylating transgenic DNA than are SJL or DBA/2 strains of mice (Engler et al., 1991). However, studies in transgenic mice utilizing the MMTV 3' LTR have not previously addressed the issue of level of transgene expression in different strains of mice. The present study demonstrates that levels of neu protein appear to be similar in mammary glands of virgin female FVB and F1 mice, suggesting that modulation of MMTV promoter activity may not be responsible for the increased tumor latencies observed in this study. This conclusion is further supported by the finding that mammary tumors induced in transgenic mice by the MMTV-myc and MMTV-ras transgenes are not affected by the presence of the C57Bl/6 genome (Hundley et al., 1997). In this study, the authors report that tumor latency in these transgenic mice did not significantly differ if the transgene was expressed on a pure strain FVB or an FVB x C57Bl/6 x Balb/c outbred background. Thus, while we cannot conclusively rule out a role for strain specific differences in MMTV promotor methylation, it would appear that mammary tumor induction by the over-expression of the *neu* proto-oncogene is influenced by the presence of tumor suppresser genes present in C57Bl/6 mice.

The finding that mammary tumor growth rates are increased in F1 mice compared with pure strain FVB mice is of interest. As tumors from F1 and FVB mice express similar levels of the neu protein, changes in the level of *neu* expression do not appear to underlie this phenomena. One possible explanation of this finding is that F1 mice are considerably older that pure strain FVB mice when their mammary tumors arise and age related loss of suppressive influences from the surrounding normal tissues could occur in F1 mice. Alternately, it is possible that genetic changes required to induce mammary tumors in F1 mice result in the development of a more aggressive tumor phenotype. In support of this hypothesis, we have demonstrated that F1 mice preferentially lose the B6 allele of chromosomes 3 and 4 (Ritland et al.,1997). As chromosome 4 has been reported to contain a number of tumor suppresser genes, it is possible that loss of these genes results in a more aggressive tumor.

Future Directions:

The studies described in this report utilize the unique strength of the *Muc-1* mutant mouse model to investigate the role of the Muc-1 molecule in organogenesis, tumor development and progression and in tumor metastasis. These are the first studies to directly demonstrate a role for Muc-1 overexpression in facilitating the growth of breast cancer *in vivo*. It is hoped that in the long term the data derived from these studies could be used to improve the treatment of human breast cancer. Data also demonstrated C57Bl/6 mice contain genetic elements that suppress the ability of *neu* proto-oncogene overexpression to induce mammary tumors in *neu* transgenic mice. It is likely that FVB mice contain mutant or defective copies of these genes, thus allowing *neu*-induced tumor development to occur at a more rapid rate. The identification of these tumor suppresser genes could be of great importance as the *neu* gene is over-expressed in approximately 25% of human breast cancers.

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Figure Legends:

Figure 1. Northern Analysis of *Muc1* Expression in Wild Type and Muc1 Deficient Mice. Approximately equivalent amounts of total RNA isolated from inbred 129svj +/+, +/- and -/- mice were size-fractionated through a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. RNA was hybredized with the mouse Muc1 cDNA probe, pMuc2TR. Below is an image of the membrane after staining with methylene blue to detect ribosomal RNAs prior to hybredization. The position of the 18s ribosomal RNA subunit is indicated.

Figure 2. Immunohistochemical Investigation of Muc1 Expression in Wild Type and Muc1 Deficient Mice.

Tissues from +/+, +/- and -/- mice were isolated, fixed and sectioned. Sections were incubated with the polyclonal rabbit antiserum to the Muc1 cytoplasmic tail (CT1) or antiserum previously blocked with immunizing peptide, followed by flourescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins. Equivalent sections were viewed and photographed under identical conditions.

Figure 3. Mammary Gland Organogenesis in Muc-1 Deficient and Wild Type Mice. Muc-1 deficient and wild type virgin female mice were terminated at 4, 5 and 6 wk. Mammary glands were removed, spread on glass slides, fixed in acetone overnight and stained with Mayer's hematoxylin. The extent of invasion of ductal elements into the mammary fat pads was assessed. At 4 wks of age, ductal development was significantly greater in Muc1 deficient mice (n= 13) than that observed in wild type mice (n= 14). By 5 wks, there were no differences in ductal penetration of the mammary fat pad between Muc-1 deficient (n= 7) and wild type (n= 8). Bars represent mean ± standard error margin.

Figure 4. Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth in Muc-1 Mutant and Wild Type Mice.

A, hematoxylin-eosin-stained sections of tumors taken at 124 days, showing poorly differentiated adenocarcinomas. B, expression of Muc1 protein was assessed by immunofluorescent staining

with a polyclonal antiserum directed against the Muc1 cytoplasmic tail. Tumors in Muc1 +/+ mice expressed high levels of the Muc1 protein. *Bar* (*panels* A and B) = 100um. C, graph showing growth rate of polyoma virus middle T antigen-induced mammary tumors in Muc-1 deficient (filled squares, n=81) and wild type (open circles, n=35) mice. At 104 days of age, Muc-1 deficient mice had significantly smaller tumors than did wild type mice ($p<0.05$). By the 124 day end point, differences in tumor size were highly significant ($p<0.001$). Asterisks indicate statistical significance.

Figure 5. Rates of Proliferation of Cells in Mammary Tumors From Muc-1 Deficient and Wild Type Mice.

Muc-1 deficient and wild type virgin female mice (n=10 per group), transgenic for the polyoma virus middle T antigen were terminated when tumors reached a weight of 1 gram. On termination day, mice were injected with bromodeoxyuridine 2 h prior to termination, tumors were removed, fixed in methacarn, paraffin embedded and sectioned for immunohistochemistry. Tumor sections were stained for incorporation of BrdU using an anti-BrdU antibody and positive cells imaged with a horse radish peroxidase conjugated secondary antibody followed by incubation with diaminobenzoic acid. Cells positive for BrdU incorporation and total cells were enumerated in 5 randomly chosen fields at 400X magnification using a 25 square $1mm^2$ reticule for each tumor. There were no detectable differences in rates of tumor cell proliferation between Muc-1 deficient and wild type mice.

Figure 6. Relative Degree of Apoptosis and Necrosis in Mammary Tumors From Muc-1 Deficient and Wild Type Mice.

Tumor sections (from animals described in Figure 2) were stained using the TUNEL assay. The level of apoptosis and necrosis in each specimen were rated on a 4 point scale and all observations were confirmed by two independent observers. Mammary tumors induced in Muc-1 deficient and wild type mice did not differ in the levels of apoptosis or necrosis present.

Figure 7. Proliferation of Muc-1 Deficient and Wild Type Mammary Tumor Cells In Vitro. Mammary tumors developing in Muc-1 deficient and wild type mice transgenic for the polyoma virus middle T antigen were placed in long term culture. Tumor cells were plated in 6 well plates at 5 x 10^4 cells/well. At various times post plating, tumor cells were dissociated from dishes and counted on a hemocytometer under phase contrast. In three separate experiments, tumor cells which did not express the Muc-1 protein grew equally as well as tumors from wild type mice which express the Muc-1 protein. Cell counts for each time point were performed in triplicate and standard deviations did not exceed 10%. Tumors from wild type mice were shown to express Muc-1 under the culture conditions used in this study.

Figure 8. Effect of Injection of NK1.1 Antibody on Natural Killer Cell Activity. In vitro measurement of NK cell activity following ip injection of 75ul of monoclonal antibody NK1.1 ascites. (A) Mice were injected with either PBS or NK1.1 antibody and terminated at the time points indicated (3 mice/group/timepoint) and the ability of splenic lymphocytes to lyse [^{51}Cr] labelled Yac1 target cells in a 6 hr cytotoxicity assay at an effector to target cell ratio of 150:1. A single injection of NK1.1 antibody (filled square) resulted in a sustained suppression of NK cell activity for 7 days relative to PBS injected control mice (circles). (B) The ability of splenic lymphocytes to lyse [^{51}Cr] labelled Yac1 target cells in a 6 hr cytotoxicity assay at an effector to target cell ratio of 150:1 was measured in mice injected with PBS (n=2), injected one time with NK1.1 antibody (n=1) or injected weekly with NK1.1 antibody for 3 months (n=3). Mice were injected 3 days prior to termination and measurement of NK cell activity. This assay demonstrates the ability of repeated injections of NK1.1 to maintain the suppression of NK cell activity for up to 3 months.

Figure 9. Role of Natural Killer Cells in Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth.

Rate of polyoma virus middle T antigen-induced mammary tumor growth in Muc-1 deficient (open symbols) and wild type (filled symbols) mice. Virgin female mice were weaned at 3 weeks of age and housed in groups of 4 to 5 mice per cage. Beginning at 4 weeks of age, mice

were injected with either 75ul of NK1.1 ascites (circles) or with PBS (squares). Tumor growth was followed on a weekly basis until the mice were 121 days old. Tumor growth was not significantly different between NK1.1 and PBS-injected mice in either Muc-1 deficient or wild type mice.

Figure 10. The Effect of Genetic Background on Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth.

Mammary tumor growth was followed for 80 days in polyoma virus middle T antigen transgenic mice of pure strain FVB (n=22, square), FVB x 129sv F1 (n=31, circle) or FVB x C57Bl/6 F1 (n=11, triangle). Tumor growth rate was not significantly different between FVB and FVB x 129sv F1 mice, while tumors in FVB x C57Bl/6 mice grew significantly more slowly than did tumors in FVB mice ($p<0.01$).

Figure 11. Reanalysis of Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth in Muc-1 Mutant and Wild Type Mice.

To correct for possible influences of variations in genetic backgrounds of mice in the original study (figure 1), mice that exhibited rapid tumor growth rate patterns indicative of the pure strain FVB (or 129sv) genetic background were removed from the data set and the data were reanalyzed. Animals were removed from the data set if their tumors displayed signs of rapid early growth; tumor burden exceeded 0.3 grams at 90 days of age (11 Muc-1 deficient mice and 8 wild type mice). On reanalysis of the data, tumor growth was still significantly slower in Muc-1 deficient mice (filled square, n=70) than in wild type control mice (open circle, n=27) at 124 days of age (Figure 6, $p<0.01$).

Figure 12. MTag-Induced Mammary Tumor Lung Metastasis in Muc-1 Deficient and Wild Type Mice.

Percentage of Muc1 deficient and wild type mice with metastatic lesions in the lung at 124 days as detected by the observation of fixed lungs under a dissecting microscope.

Figure 13. Lung Metastasis in Muc-1 Deficient and Wild Type Mice Injected with NK1.1 Antibody or Vehicle.

Rates of MTag-induced mammary tumor metastasis were compared between Muc-1 deficient and wild type control mice treated with either anti-NK antibodies (NK1.1) or vehicle (n=20 mice per group). Mice were injected with NK1.1 or PBS at weekly intervals beginning at 4 weeks of age. Mice were terminated at 121 days of age and their lungs removed, fixed in methacarn and examined under a dissecting microscope for lung metastasis. Depletion of NK cell activity did not result in a change in the rate of metastasis in either Muc-1 deficient or wild type mice

Figure 14. Tumor Incidence in Neu Proto-Oncogene Transgenic Female Mice

A, Kapplen-Meier plot of tumor latency in *neu* transgenic mice. Inbred FVB virgin female mice transgenic for the neu proto-oncogene developed mammary tumors between 7 and 12 months of age with a 70% incidence at 12 months of age. In contrast, at 18 months of age only 5% of FVB x C57Bl/6 F1 transgenic females developed mammary tumors. When F1 mice were backcrossed onto inbred FVB mice (F1B1), neu transgenic females developed mammary tumors with approximately a 35% incidence at 14 months. B, scatter plot of tumor latencies in *neu* proto-oncogene transgenic female mice.

Figure 15. Expression of the Neu Proto-Oncogene in Mammary Glands of Virgin Female Mice.

To compare the expression levels of the neu proto-oncogene in the normal mammary glands FVB and FVB x C57Bl/6 F1 mice, neu transgene positive virgin female mice from each condition (n=2) were housed together for 1 month to allow for synchronization of estrous cycles. Animals were terminated at 3 months of age and mammary glands were removed, fixed in methacarn and sectioned for immunohistochemistry. Tissue sections were stained with anti-neu antibody. Although expression levels varied in all glands examined, both FVB and FVB x C57Bl/6 F1 mice exhibited strong staining of some ductal elements.

Figure 16. *Neu* Proto-Oncogene-Induced Mammary Tumor Growth Rate in FVB and FVB x C57Bl/6 F1 Mice.

Inbred FVB and FVB x C57Bl/6 F1 female mice transgenic for the *neu* proto-oncogene (n=10 per group) were palpated bi-weekly for the development and growth of mammary tumors. Day 0 was defined as the last palpation before tumor growth was noted. To directly compare tumor growth rate in these two groups, the data was logarithmically transformed and a best fit linear regression was estimated for each tumor. When the slopes of these lines were compared, tumors in F1 mice grew significantly faster than tumors growing in pure strain FVB mice ($p<0.01$)

Figure 17. Expression of the Neu Proto-Oncogene in Mammary Tumors.

Levels of expression of the neu proto-oncogene in mammary tumors from FVB and FVB x C57Bl/6 F1 mice were compared by immunohistochemistry (A) and by Western blot analysis (B). All tumors examined expressed high levels of the neu protein.

FIGURE 1

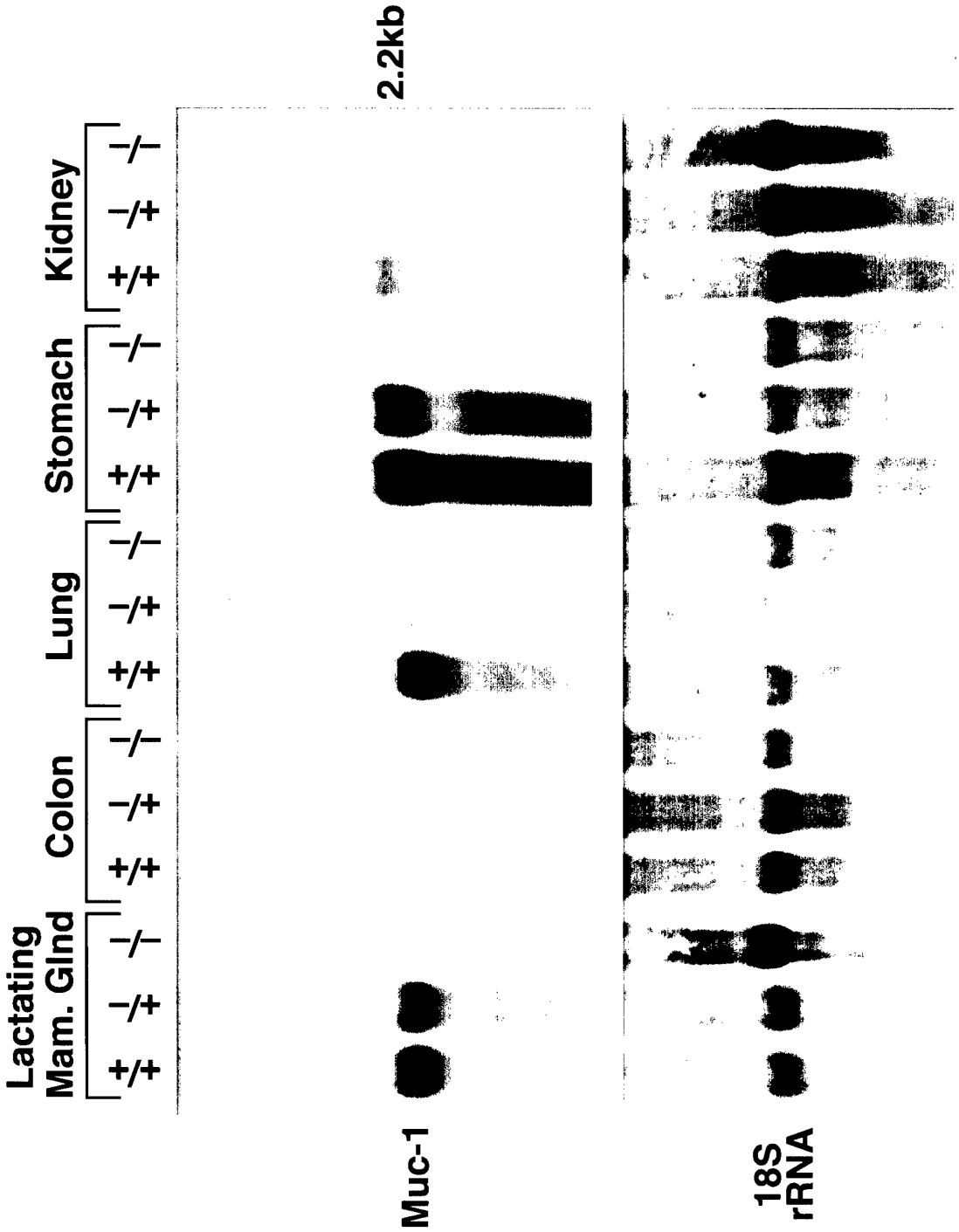

2.2kb
Lactating Mam. Glnd
Colon
Lung
Stomach
Kidney
−/−
−/+
+/+
Muc-1
18S rRNA

FIGURE 2

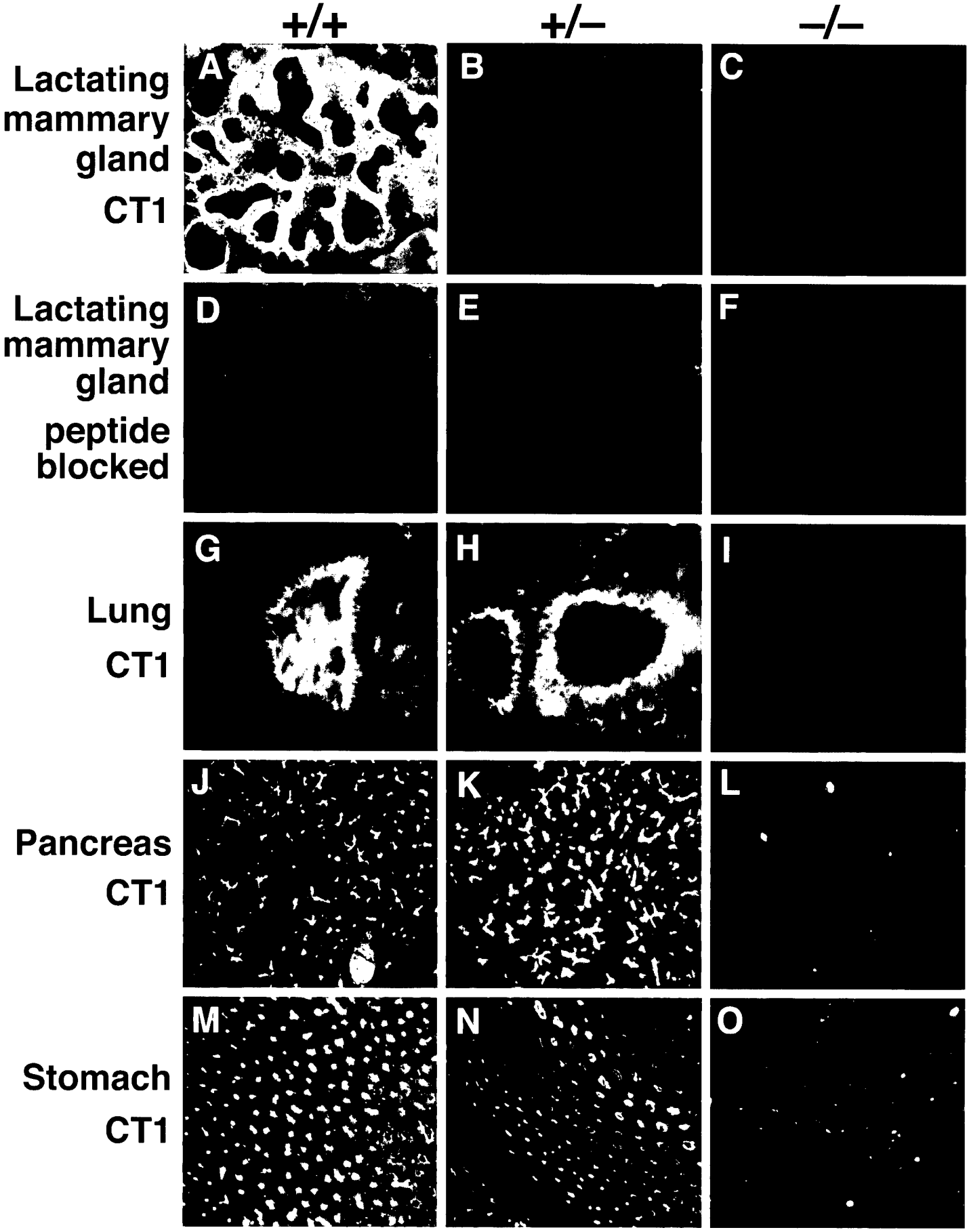

+/+
+/–
–/–
Lactating mammary gland CT1
A
B
C
Lactating mammary gland peptide blocked
D
E
F
Lung CT1
G
H
I
Pancreas CT1
J
K
L
Stomach CT1
M
N
O

FIGURE 3

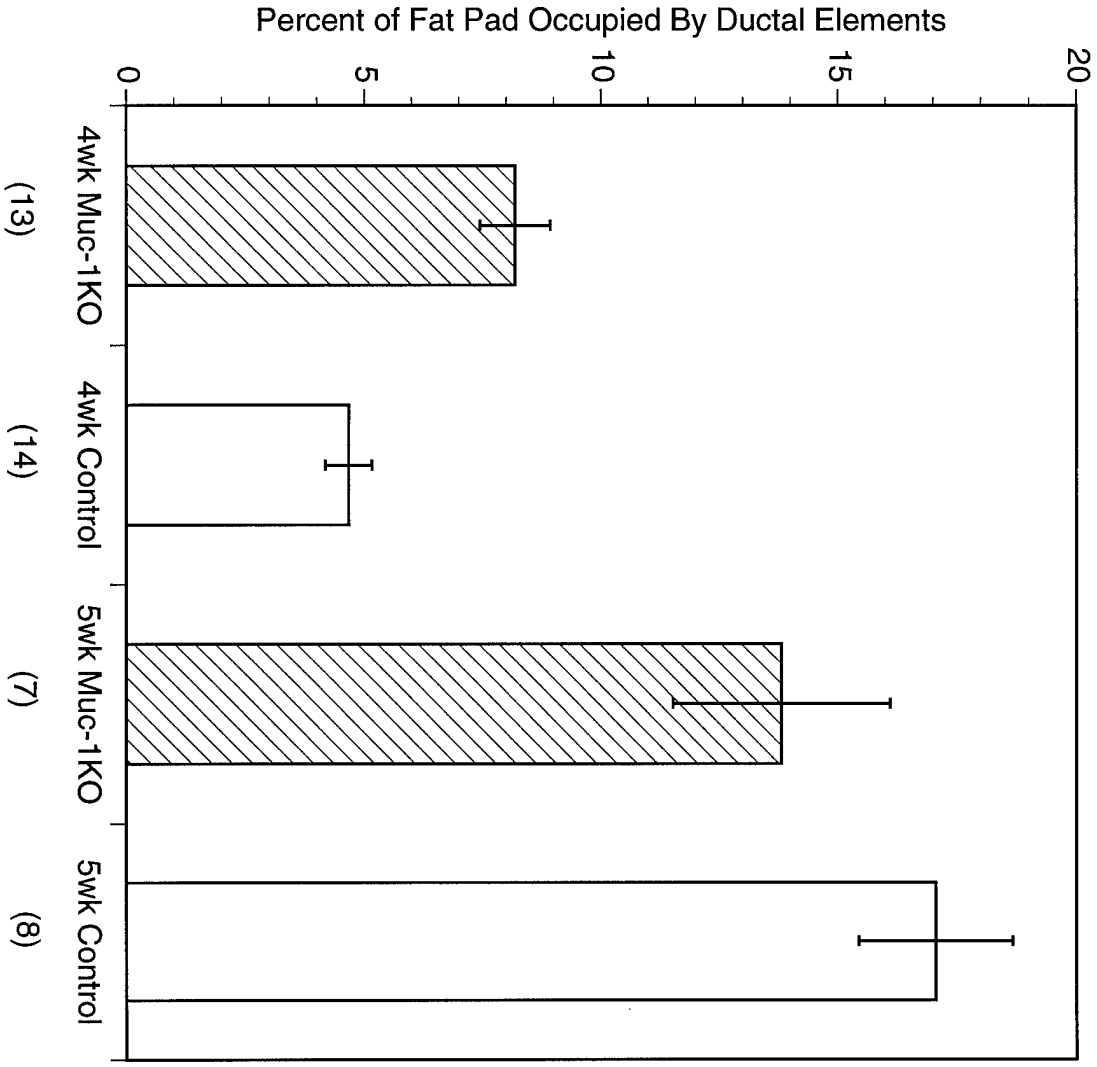
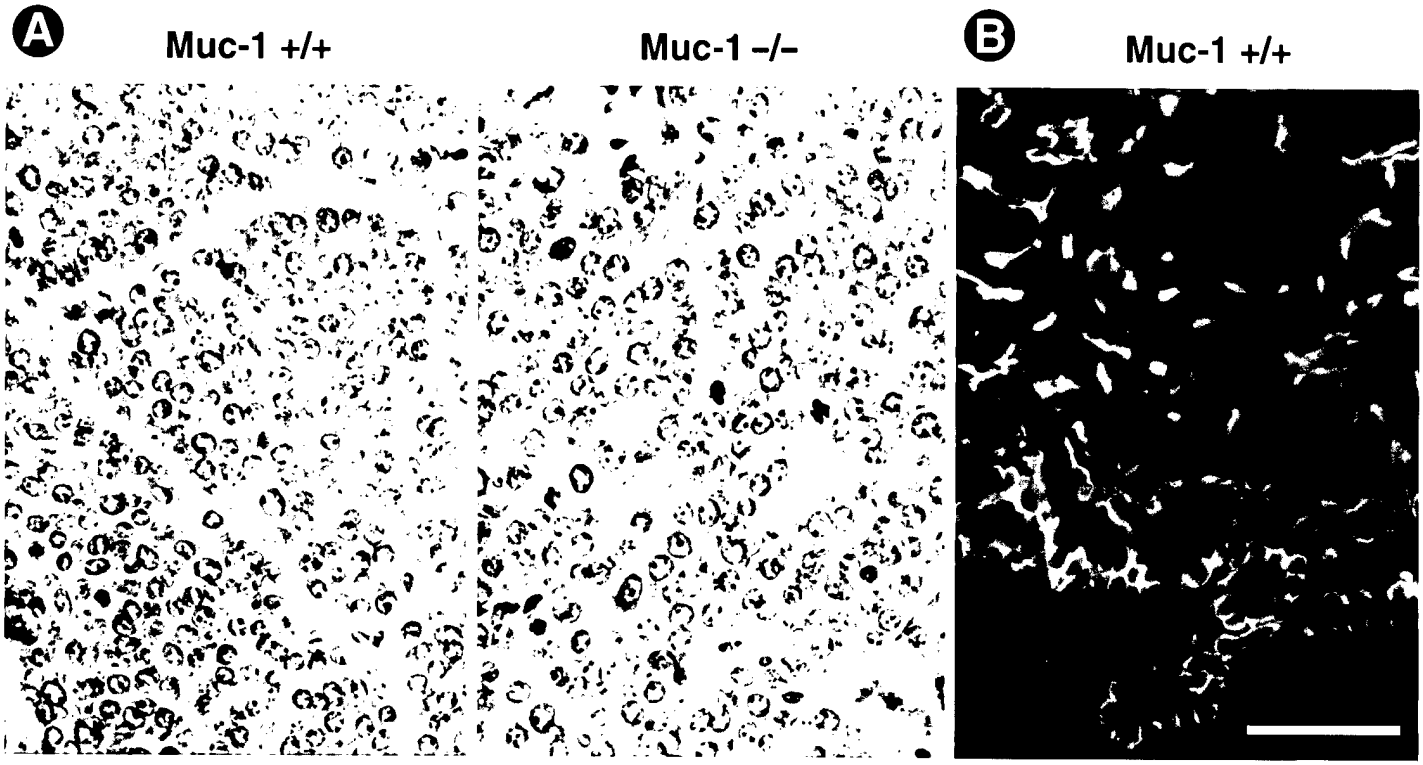

Percent of Fat Pad Occupied By Ductal Elements
0
5
10
15
20
4wk Muc-1KO
(13)
4wk Control
(14)
5wk Muc-1KO
(7)
5wk Control
(8)

FIGURE 4


A
Muc-1 +/+
Muc-1 -/-
B
Muc-1 +/+

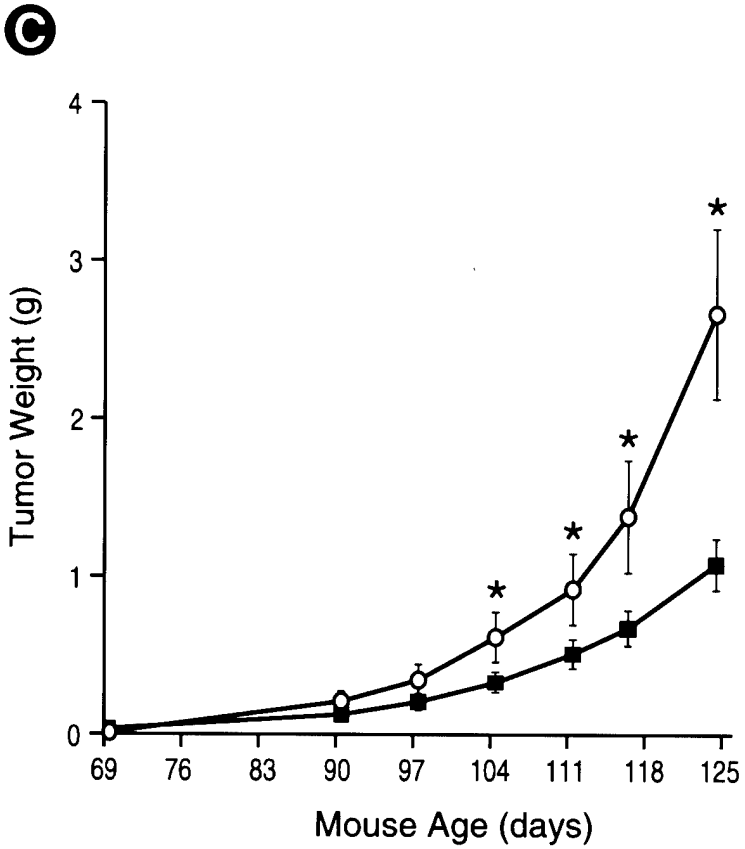

C
Tumor Weight (g)
0
1
2
3
4
69
76
83
90
97
104
111
118
125
Mouse Age (days)
*
*
*
*

FIGURE 5

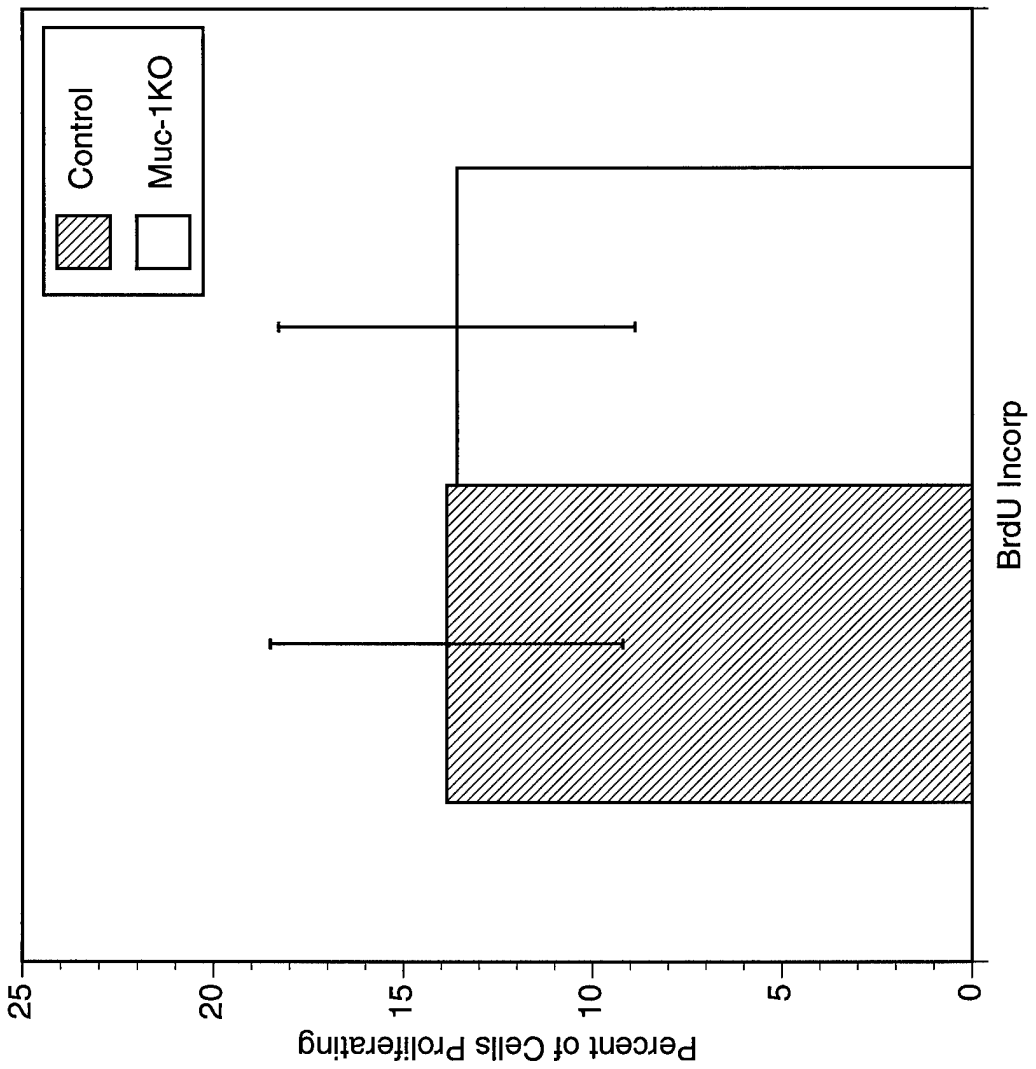

Control
Muc-1KO
Percent of Cells Proliferating
0
5
10
15
20
25
BrdU Incorp

FIGURE 6

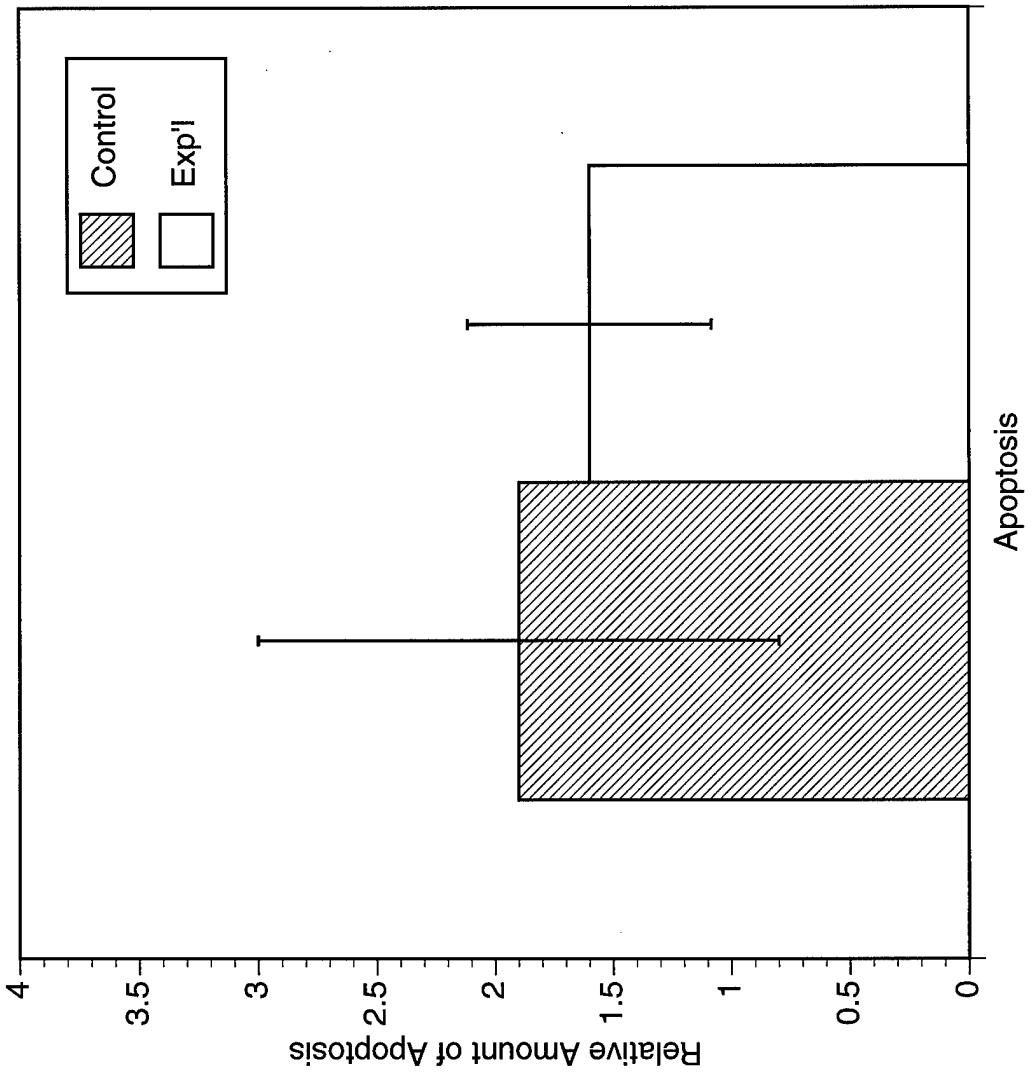
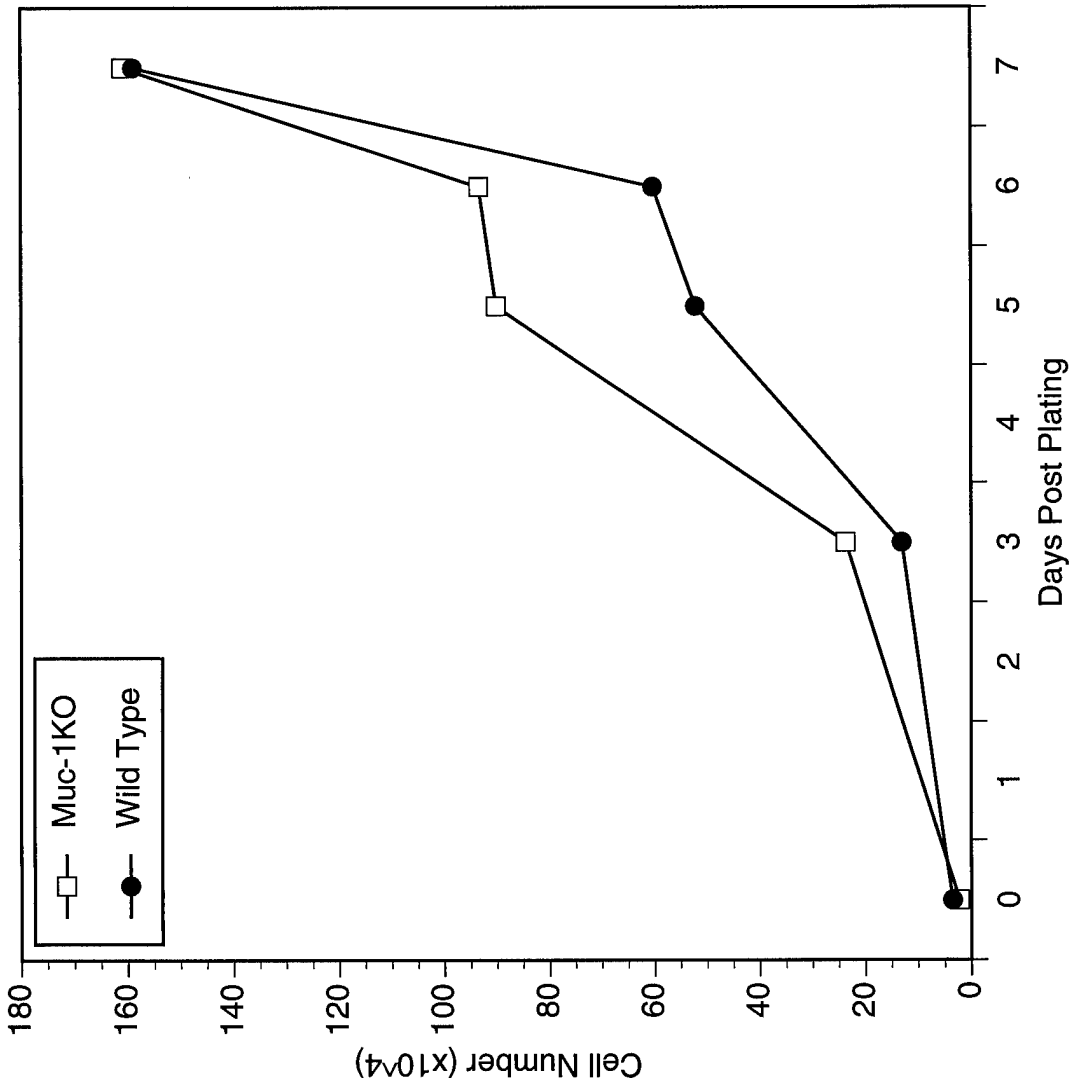
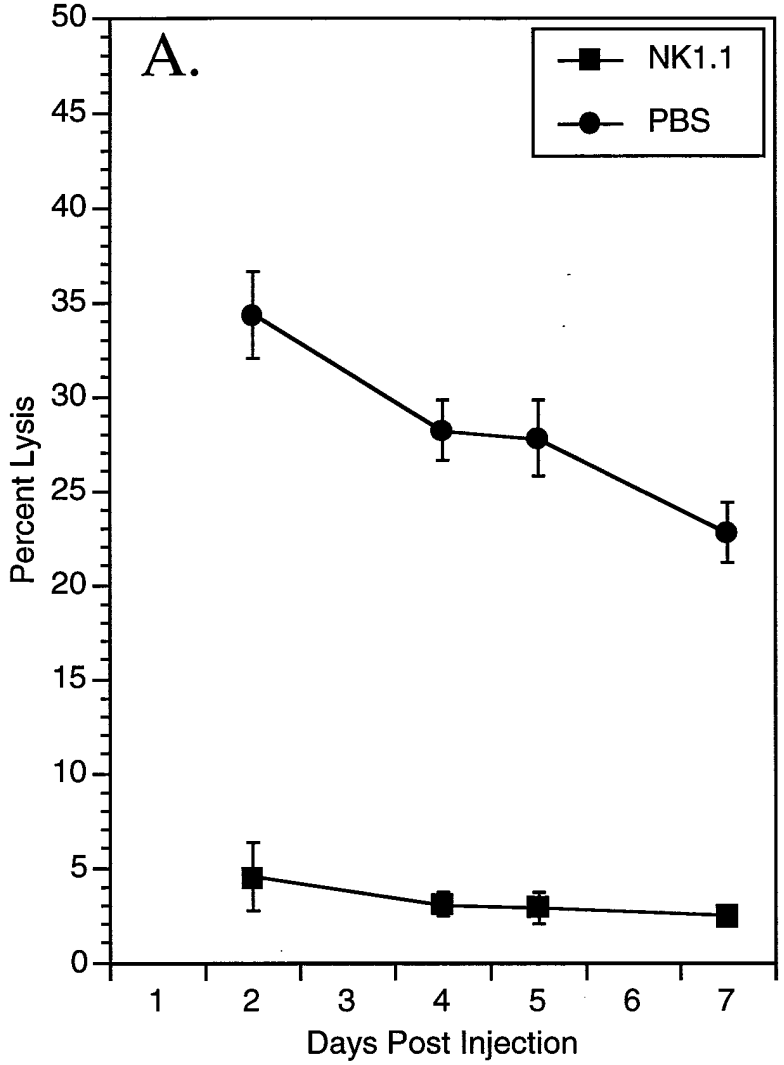

Control
Exp'l
Relative Amount of Apoptosis
0
0.5
1
1.5
2
2.5
3
3.5
4
Apoptosis

FIGURE 7


Muc-1KO
Wild Type
Cell Number (x10^4)
Days Post Plating
0
20
40
60
80
100
120
140
160
180
0
1
2
3
4
5
6
7


A.
NK1.1
PBS
Percent Lysis
Days Post Injection
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7

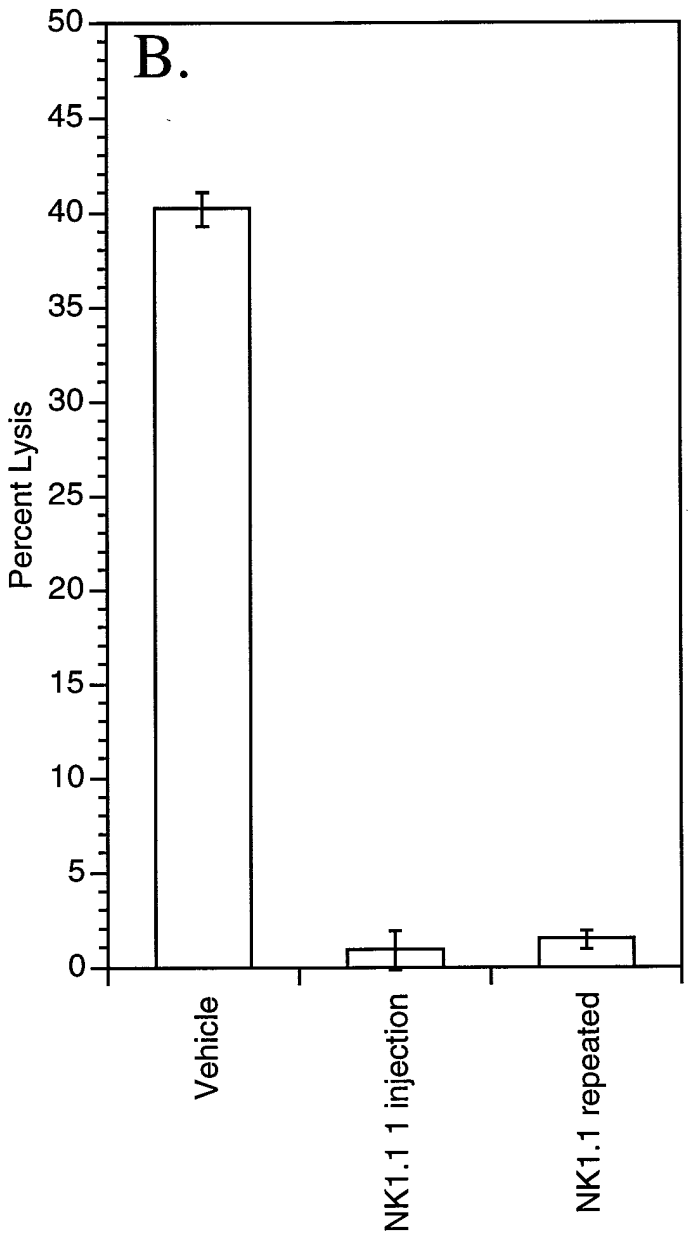

B.
Percent Lysis
0
5
10
15
20
25
30
35
40
45
50
Vehicle
NK1.1 1 injection
NK1.1 repeated

FIGURE 8

FIGURE 9

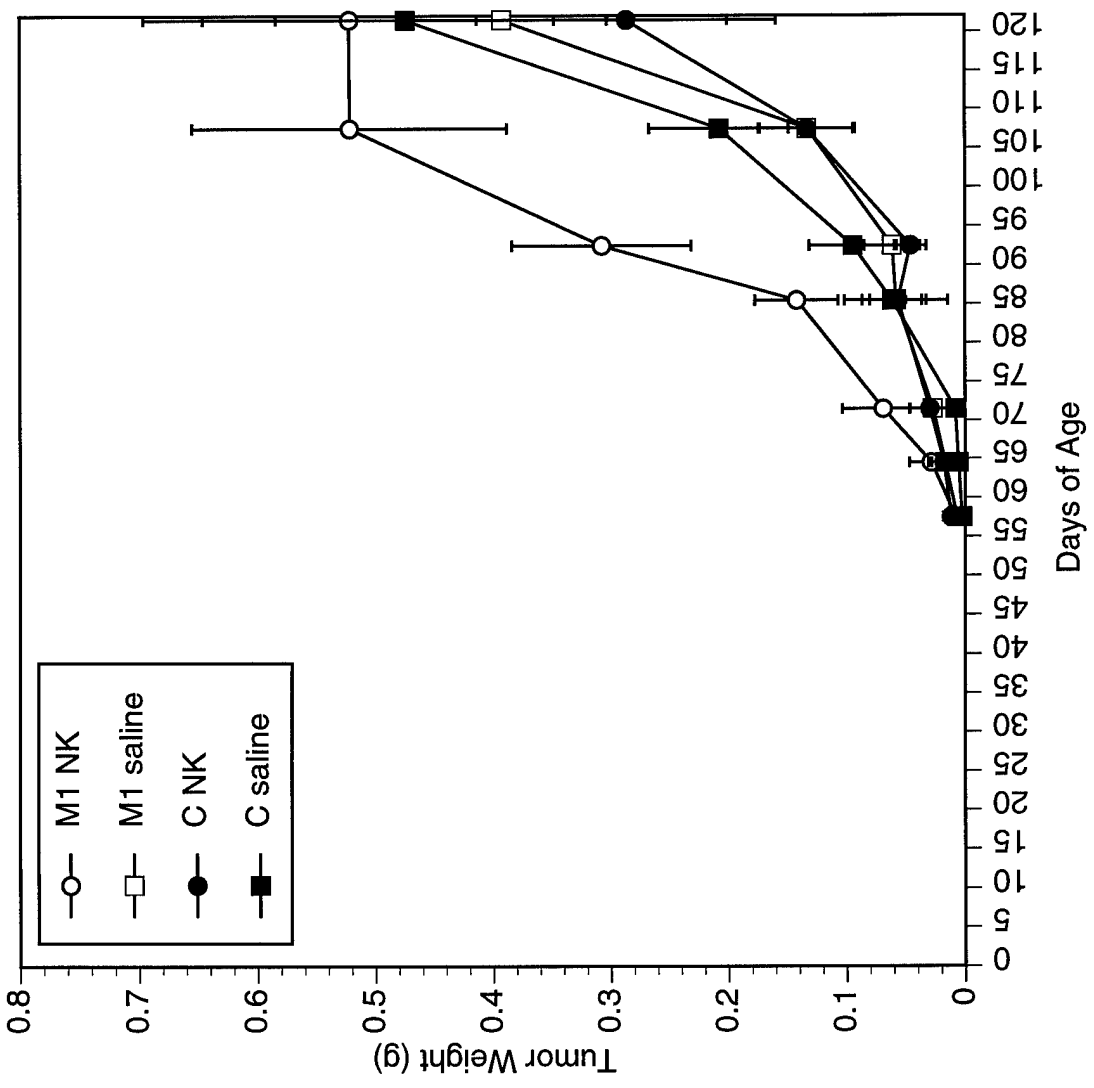

Tumor Weight (g)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Days of Age
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
M1 NK
M1 saline
C NK
C saline

FIGURE 10

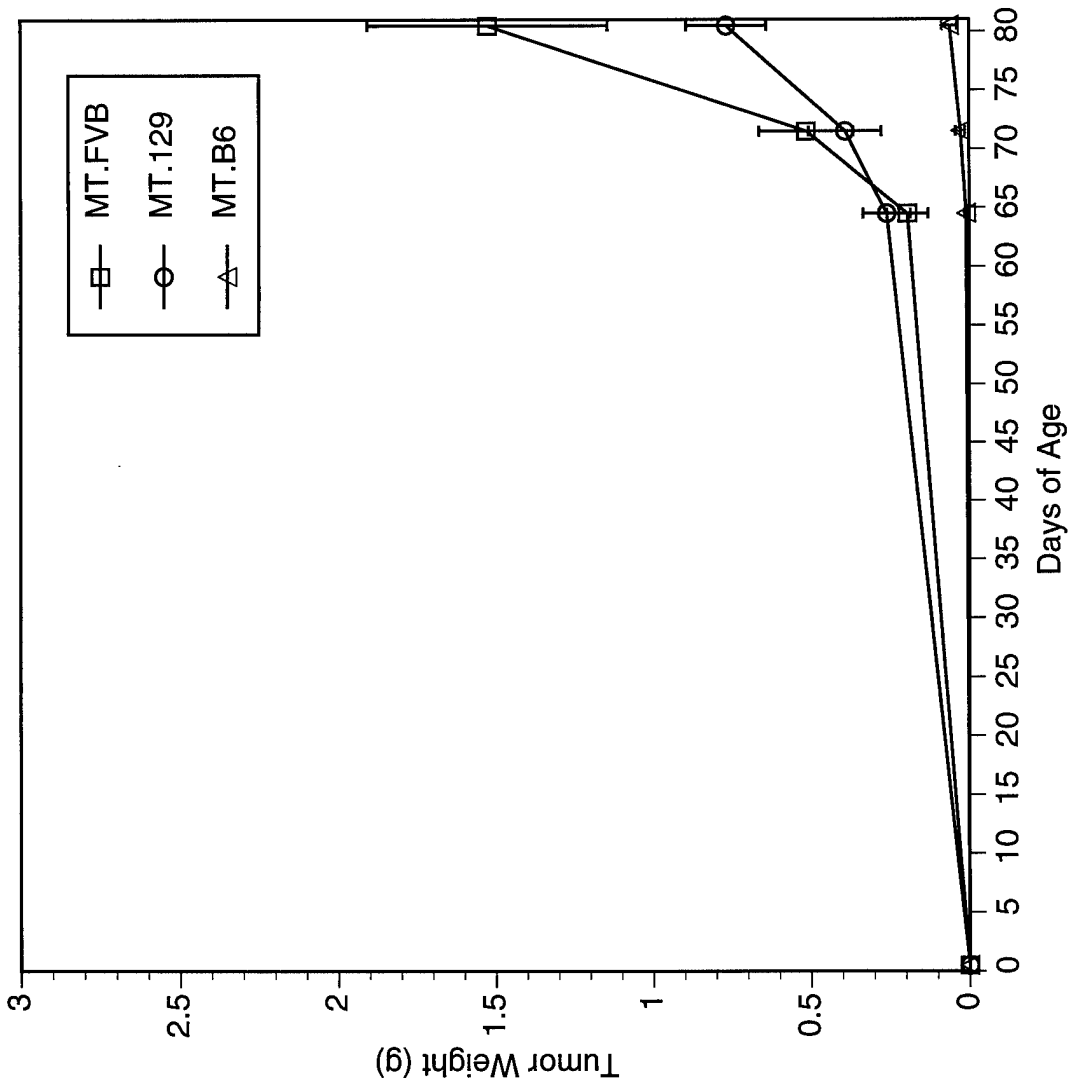

MT.FVB
MT.129
MT.B6
Tumor Weight (g)
0
0.5
1
1.5
2
2.5
3
Days of Age
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80

FIGURE 11

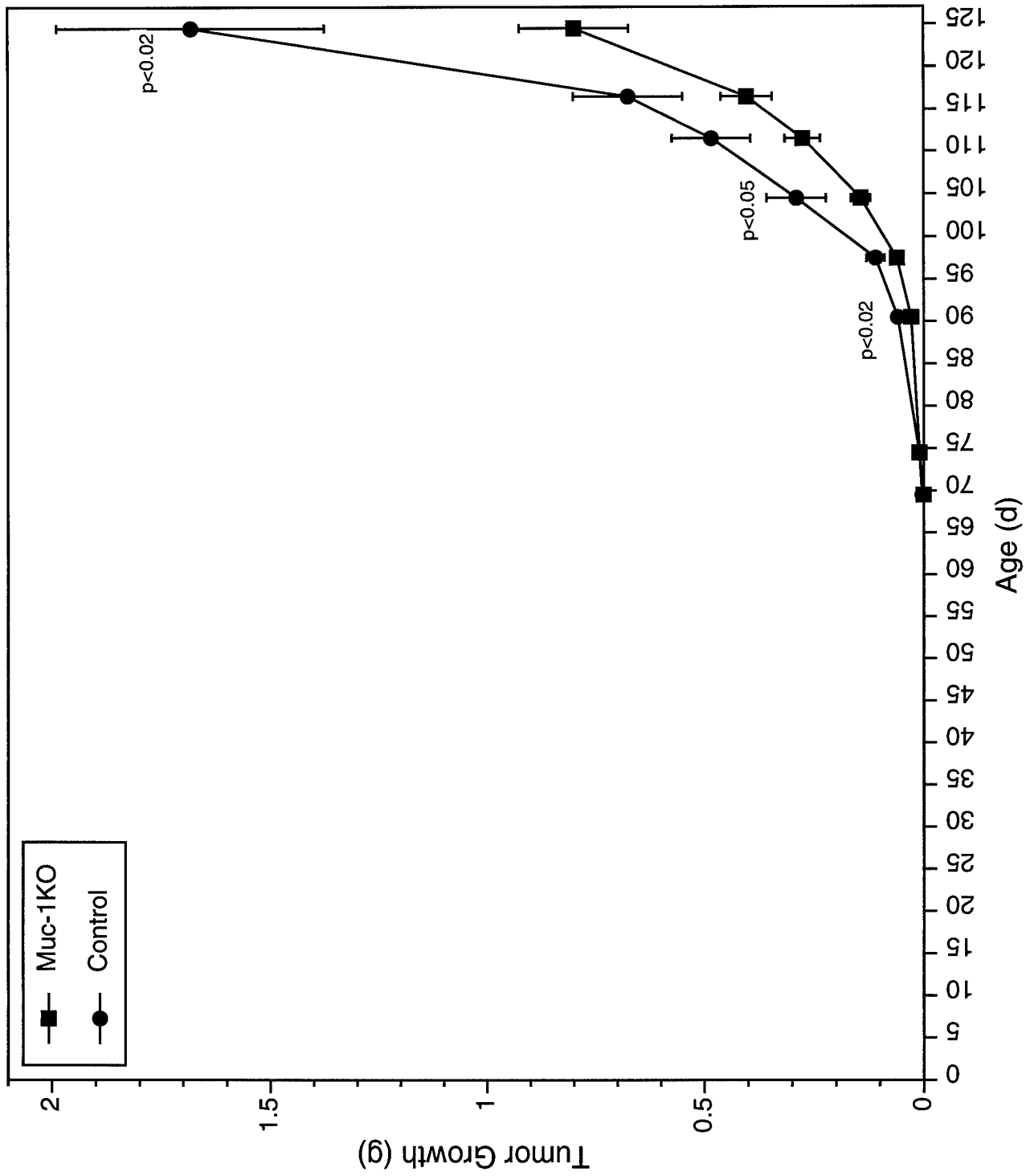

Tumor Growth (g)
0
0.5
1
1.5
2
Age (d)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
p<0.02
p<0.05
p<0.02
Muc-1KO
Control

FIGURE 12

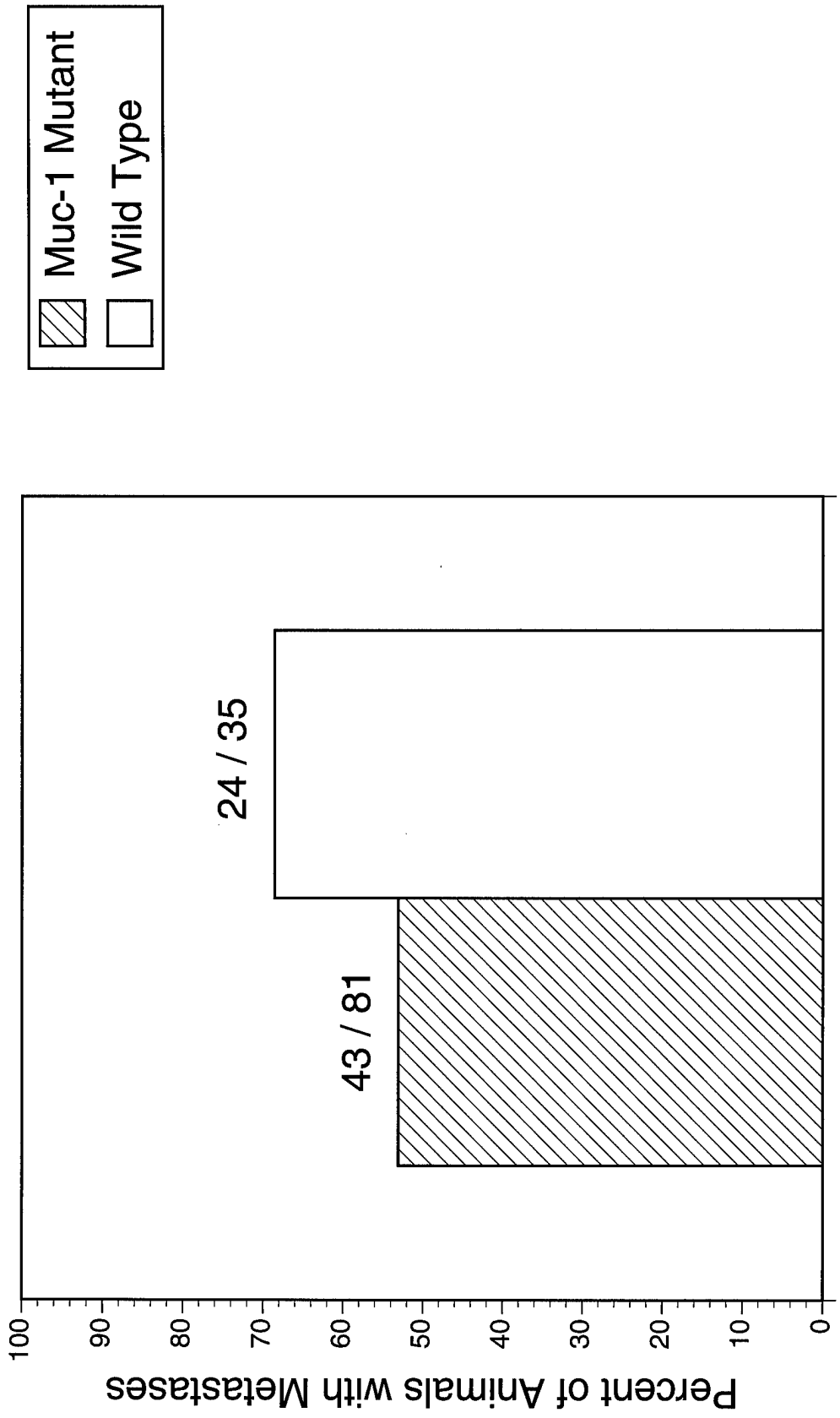
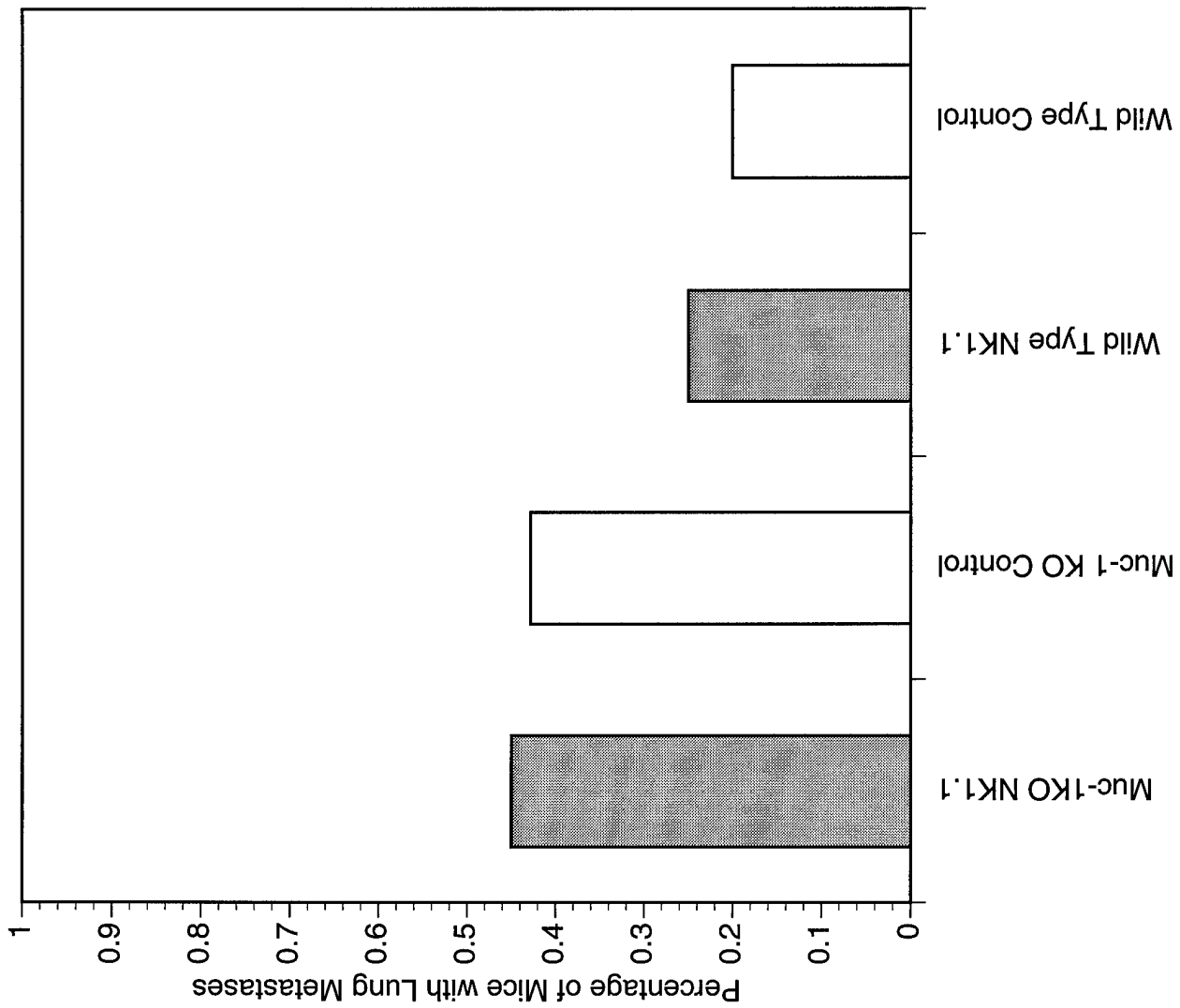

Muc-1 Mutant
Wild Type
Percent of Animals with Metastases
100
90
80
70
60
50
40
30
20
10
0
24 / 35
43 / 81

FIGURE 13


Percentage of Mice with Lung Metastases
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Muc-1KO NK1.1
Muc-1 KO Control
Wild Type NK1.1
Wild Type Control

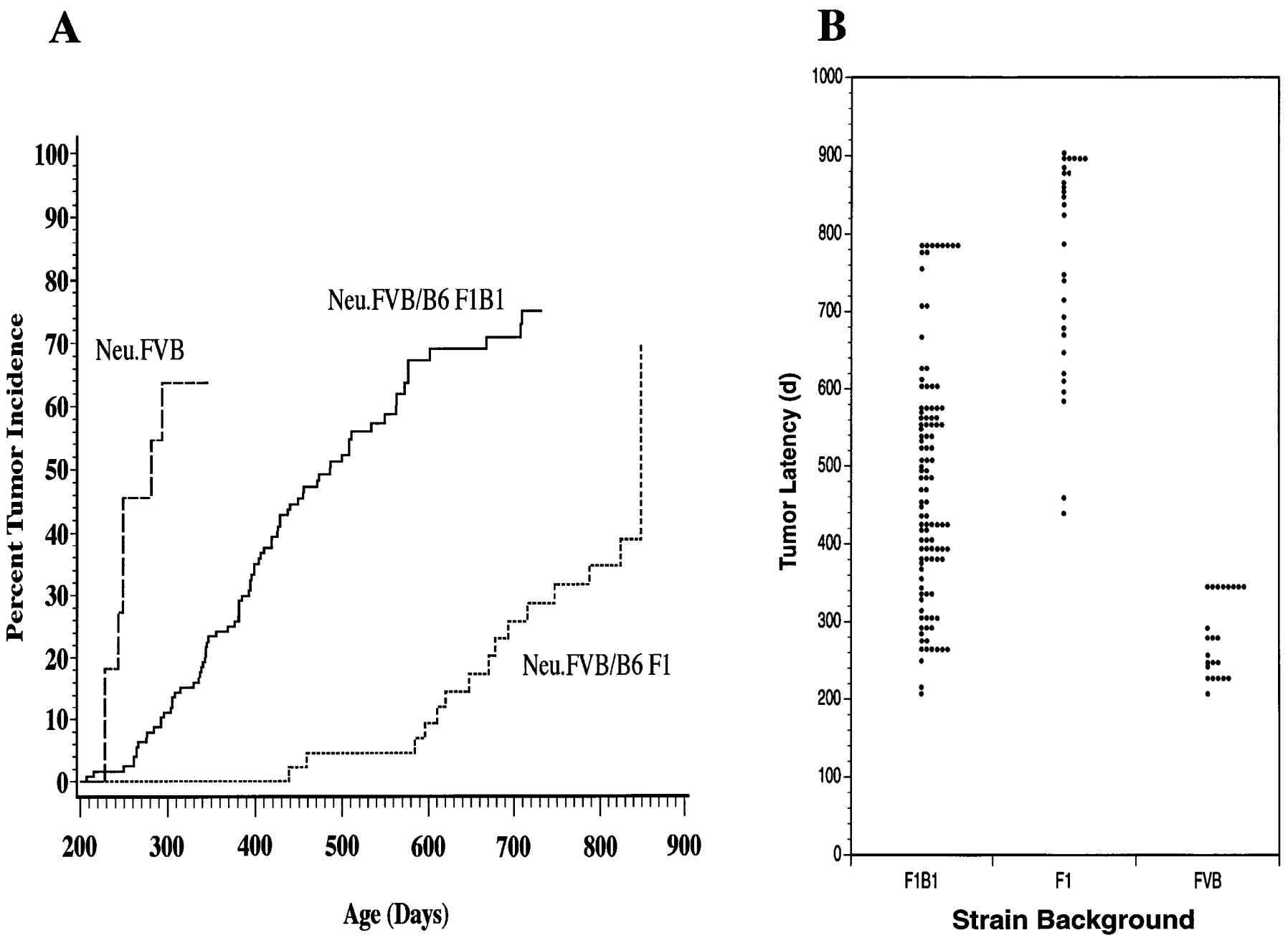

A
Percent Tumor Incidence
100
90
80
70
60
50
40
30
20
10
0
Neu.FVB
Neu.FVB/B6 F1B1
Neu.FVB/B6 F1
200
300
400
500
600
700
800
900
Age (Days)
B
Tumor Latency (d)
1000
900
800
700
600
500
400
300
200
100
0
F1B1
F1
FVB
Strain Background

FIGURE 14

FIGURE 15

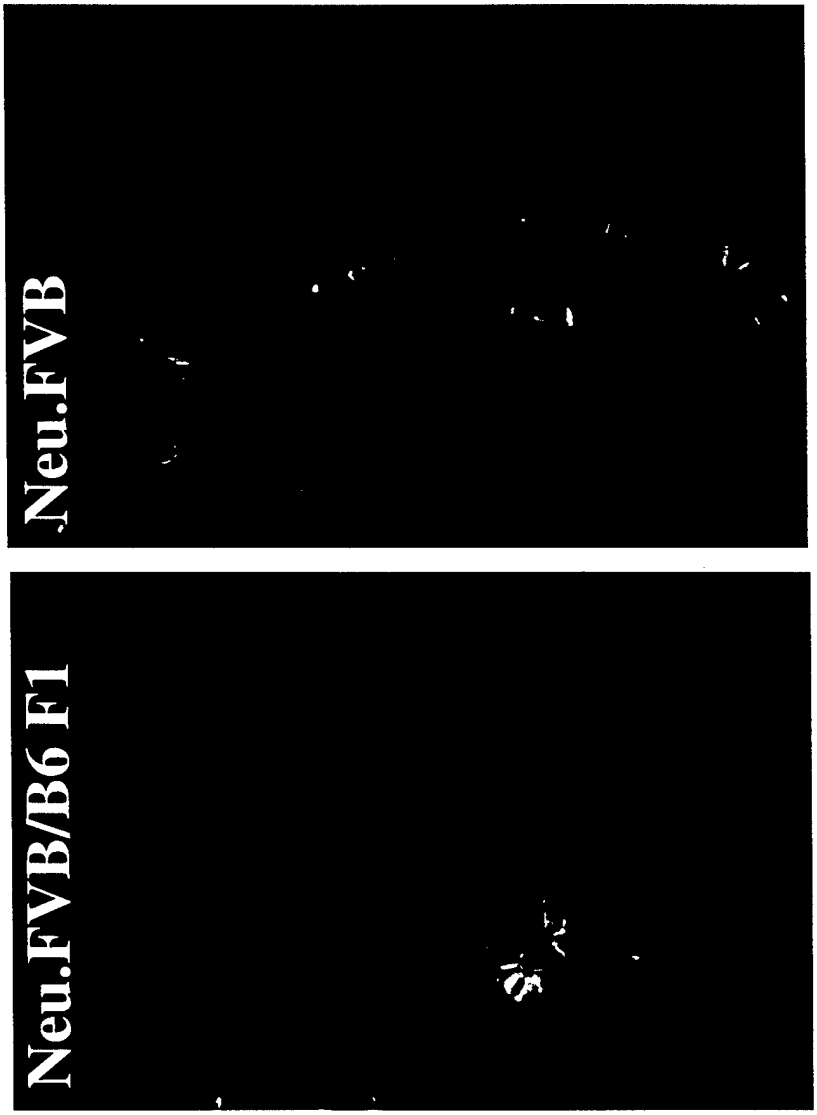
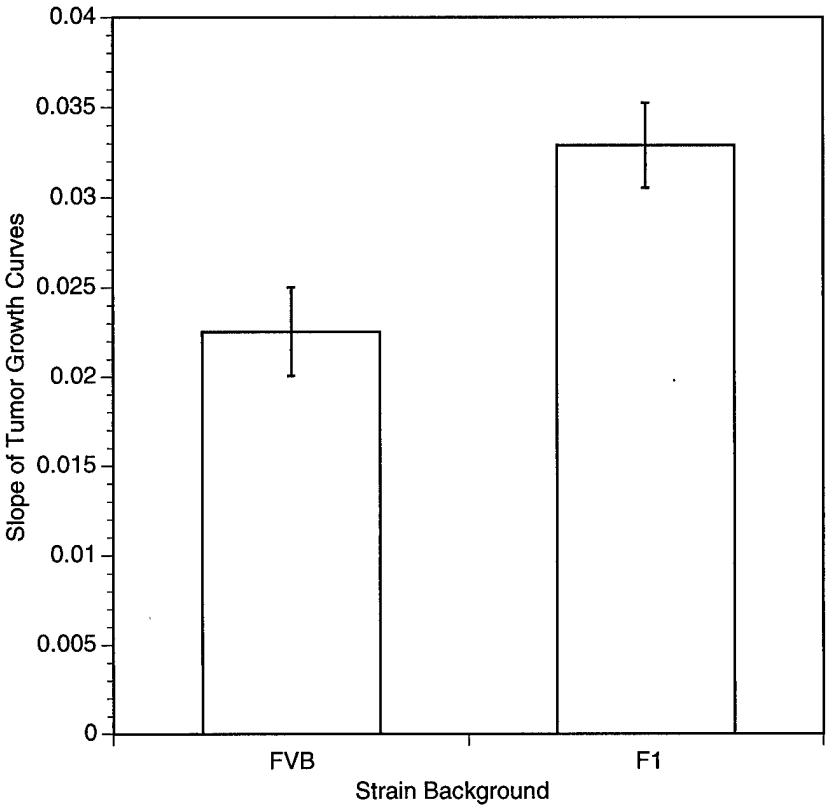
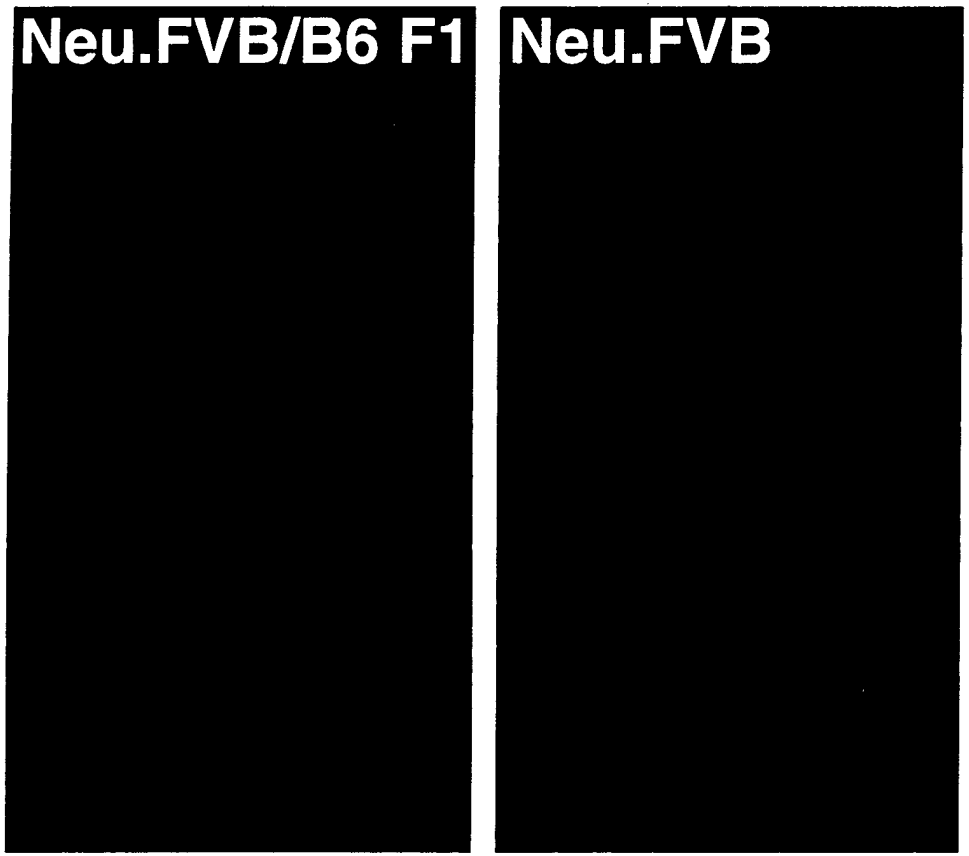


FIGURE 16


0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Slope of Tumor Growth Curves
FVB
F1
Strain Background

A



B

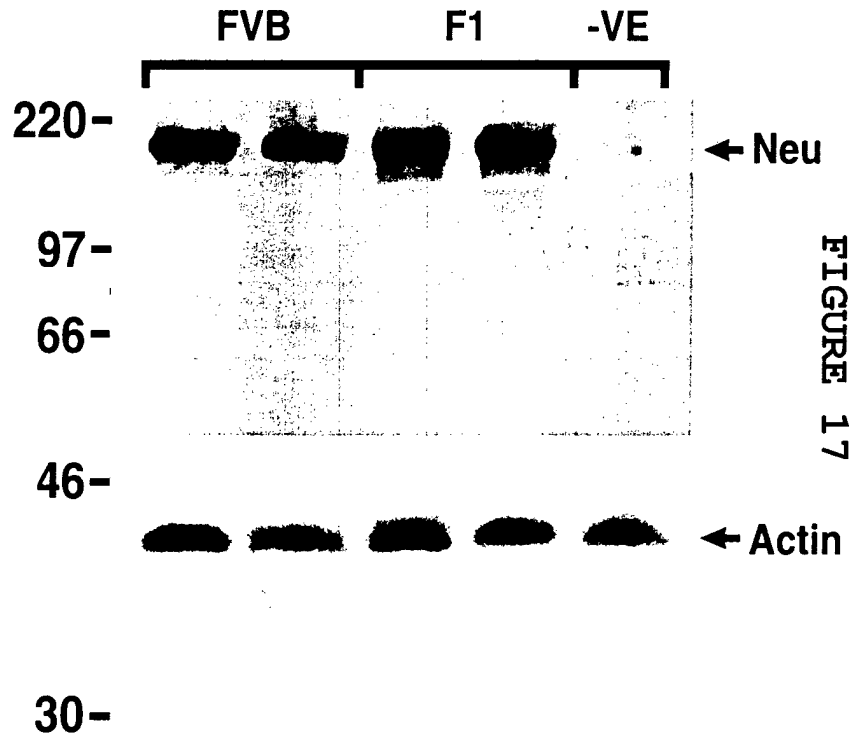


FIGURE 17

Table 1

Genes Screened for in Muc1 Deficient Mice

Gene	Species	Probe Name/Type	Locaction
Muc1	Mouse	pMuc2TR	Tandem Repeat
Muc2	Rat	VR-1A	5' cDNA
Muc4	Mouse	pMuc7.18/genomic	
Glycam-1	Mouse	Antisense-oligo	bp 468-419
MadCAM-1	Mouse	Antisense-oligo	bp 1248-1199
Glycophorin	Mouse	Antisense-oligo	bp 900-851
PSGL-1	Human	Antisense-oligo	bp 1061-1015
ASPG-2	Rat	Antisense-oligo	bp 2175-2126
CD34	Mouse	Antisense-oligo	bp 1258-1209
CD43 (Pgp-1)	Mouse	Antisense-oligo	bp 1183-1134
CD45 (Ly-5)	Mouse	Antisense-oligo	bp 1000-951
Thbs-3	Mouse	mThbs-3Eco	2.1-kb EcoRI (3')